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Review article

Ovarian function in the buffalo and implications for embryo development and assisted reproduction

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ABSTRACT

This review brings together information on ovarian physiology in buffaloes including folliculogenesis, ovulation, and the development and function of the corpus luteum. Features of embryonic development are also considered. The buffalo is classified as a short-day breeder but in equatorial zones can show oestrous cycles throughout the year provided that nutrition is adequate to maintain reproductive function. In sub-tropical zones and at higher latitudes, day length is often the major determinant of reproductive function including the occurrence of regular oestrous cycles, duration of oestrus, and the period to resumption of ovulation postpartum. Indeed, at higher latitudes buffaloes that give birth during the period of increasing day length may not show a resumption of ovulation until the following period of decreasing day length. This can have a major impact on the productive value of buffaloes and requires the development and utilisation of practical and effective assisted breeding technology for out-of-season breeding in buffaloes. Embryonic development in buffaloes occurs at a faster rate than in cattle and this has implications for the earlier establishment and functionality of the corpus luteum in buffaloes. It would appear that the interrelationships between the development of the early conceptus, corpus luteum function, uterine preparation, and maternal recognition of pregnancy, are more closely time-bound in buffaloes compared with cattle. The phase of embryonic attachment would seem to be a critical period for determining the reproductive outcome in buffaloes.

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1. Introduction

The water buffalo (*Bubalus bubalis*) is a significant livestock species of developing countries in tropical and sub-tropical environments and is also an important production animal in developed countries (Zicarelli, 1994). Buffaloes have the capacity to maintain productivity on pastures that are typically limiting to cattle production (Zicarelli, 1994). The good feed conversion efficiency of buffaloes and relatively low maintenance requirements are attributes which make them ideal in low-input, low-cost production systems (Zicarelli, 1994; Paul et al., 2002). Accordingly, buffaloes have emerged as an increasingly important source of high quality animal protein, both milk and meat (Zicarelli, 1994).

For equatorial and tropical zones in which feed availability is limiting, buffaloes can show a delay in the onset of puberty and have relatively long inter-calving intervals (Zicarelli, 2007). In sub-tropical environments and at higher latitudes photoperiod influences reproductive function in buffaloes in addition to nutrition (Zicarelli, 1989, 1997; Baruselli, 1993). The influence of photoperiod means that, without intervention, buffaloes have seasonal cycles in conception, calving and milk production. To produce milk throughout the year in order to achieve continuity of market supply it is necessary to use hormonal treatments for out-of-season breeding of postpartum buffaloes (Zicarelli, 1997). However, buffaloes that are bred out-of-season often have a greater rate of embryonic mortality than buffaloes bred during the breeding season (Baruselli et al., 1997b; Campanile et al., 2005; Campanile and Neglia, 2007).

The ovaries in buffaloes are typically smaller than in cattle (Vittoria, 1997) and buffaloes have fewer follicles in each follicular wave (Gimenes et al., 2009); the latter appears to be associated with a smaller pool of primordial follicles in buffaloes compared with cattle (Danell, 1987). Based on the differences in ovarian function between buffaloes and cattle it could be concluded that buffaloes differ fundamentally in ovarian physiology. This is supported by the reduced response in buffaloes to ovarian follicular superstimulation treatments, low recovery of embryos and oocytes, and low number of transferable embryos (Misra and Tyagi, 2007; Baruselli, 1994; Zicarelli et al., 1994a,b, 2000; Baruselli et al., 1997a, 2002; Carvalho et al., 2002). The reduced response in buffaloes to hormonal treatments can be partly explained by the use of treatments that have not been extensively evaluated in buffaloes.

This review presents a summary of ovarian physiology in buffaloes including folliculogenesis, ovulation, and the development and function of the corpus luteum. These features of ovarian function are related to embryonic development and assisted reproduction. The review also identifies gaps in knowledge of reproductive physiology that could be considered important for the development of new technologies for assisted reproduction in buffaloes.

2. Oestrous cycle

The buffalo is classified as a short-day breeder but in equatorial zones can show oestrous cycles throughout the year provided that nutrition is adequate to maintain reproductive function (Seren et al., 1995). Buffaloes become increasingly seasonally polyoestrus with distance from the equator (Zicarelli, 1997; Baruselli et al., 2001) and females that calve during the non-breeding season have an extended postpartum anoestrous period with a proportion not resuming ovulation until the following breeding season (Zicarelli, 2007). The oestrous cycle in buffaloes can vary from 16 to 28 days (Manik et al., 1994; Baruselli et al., 1997a) and in buffaloes showing regular oestrous cycles, the duration of oestrus is typically 10–20 h if animals are cycling during the breeding season (decreasing day length or equatorial zones; Gill et al., 1973; Rao et al., 1981; Vale et al., 1984). Females cycling during the non-breeding season can show variable duration of oestrus, from 2 to 72 h (Zicarelli et al., 1988a; Baruselli, 2001). Irrespective of breeding or non-breeding season, buffaloes show less intense expression of oestrus compared with cattle (Ohashi, 1994) and also less oestrous-associated mounting behaviour (Roy and Prakash, 2009). It has been suggested that the reduced intensity of oestrus in buffaloes is associated, at least in part, with relative low circulating concentrations of 17β-oestradiol (oestradiol) in comparison with dairy cattle (Seren et al., 1995). The pre-ovulatory follicle in buffaloes is smaller than in dairy cattle (Baruselli et al., 1997a) and this could be associated with lower oestradiol levels although there could also be differences in oestradiol metabolism and clearance from circulation between buffaloes and dairy cattle. The interval between the onset of oestrus and the LH surge can vary from 1 to 12 h in buffaloes (Gill et al., 1973; Rao et al., 1981; Vale et al., 1984; Seren et al., 1995) and ovulation occurs between 26 and 33 h after the LH surge (Seren et al., 1995; Porto-Filho et al., 1999).

3. Ovarian function

3.1. Follicular growth

Buffaloes show waves of ovarian follicular development during oestrous cycles similar to cattle (Baruselli et al., 1997a). The fundamental features of follicle recruitment, selection, dominance and atresia during a follicular wave in buffaloes (Baruselli et al., 1997a) are similar to those described for cattle (Sirois and Fortune, 1988; Ginther et al., 1989a; Mapletoft et al., 1994). The number of follicular waves during an oestrous cycle can vary from 1 to 3

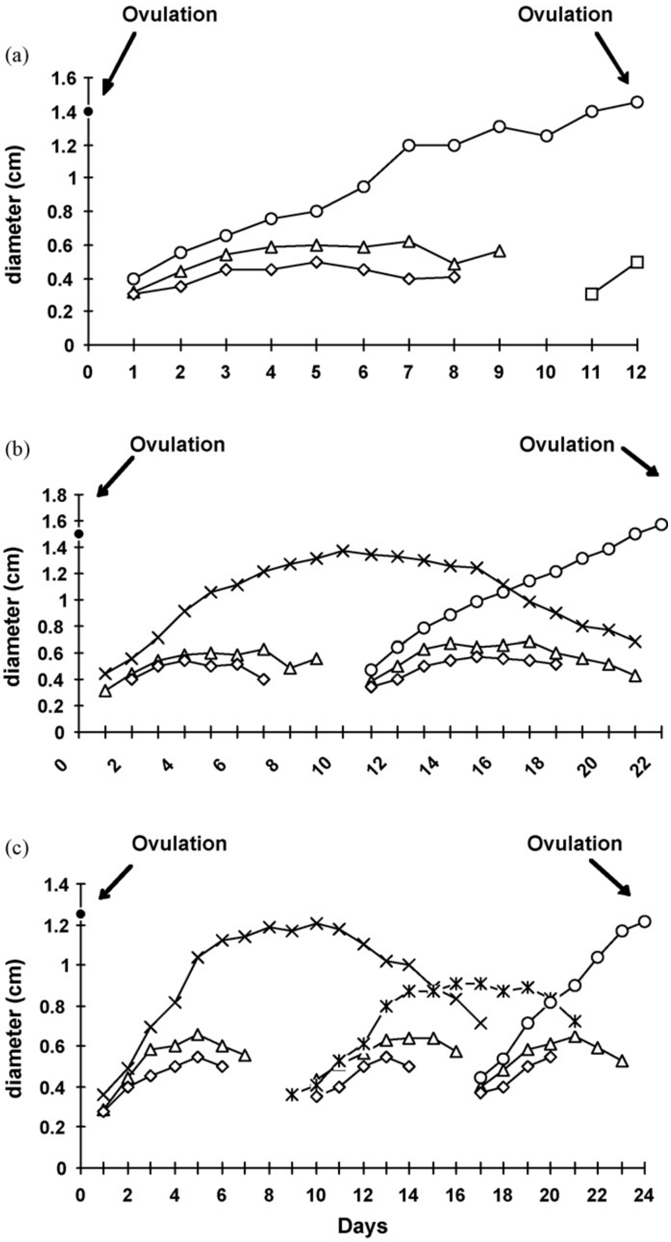

Fig. 1. Standardised diameters of ovarian follicles (○, dominant follicle; △, largest subordinate follicle; ◇, second largest subordinate follicle) in buffalo cows with (a) 1 wave, (b) 2 wave and (c) 3 wave ($n = 10$) oestrous cycles (Baruselli et al., 1997a).

in buffaloes, with 2 waves being the most common (63%; Baruselli et al., 1997a; Presicce et al., 2004; Neglia et al., 2007). There has been no report of 4 follicular waves within an oestrous cycle in buffaloes, although 4 waves do occur in cattle (Rhodes et al., 1995). In buffaloes, 3 follicular waves occur in oestrous cycles that have a longer luteal phase and greater inter-ovulatory interval compared with cycles that have 2 follicular waves (Fig. 1).

The number of follicles recruited into a follicular wave was reported to be lower in buffaloes than in cattle (Danell, 1987; Ty et al., 1989). However, in a recent study (Gimenes et al., 2009), buffalo heifers were shown to recruit a similar number of follicles as Holstein heifers (*Bos taurus*) (13.1 ± 1.4 and 15.0 ± 2.8 follicles, respectively) which was lower than the number recruited in Nelore heifers (Zebu, *Bos indicus*) (29.7 ± 3.1 follicles). Notwithstanding the latter, the relatively small number of follicles typical of follicular waves in buffaloes could be related to the smaller pool of primordial follicles reported for buffalo heifers (12,636) compared with cattle (50,000) (Danell, 1987).

Buffalo heifers also tend to have a greater rate of follicle atresia (67%; Danell, 1987) relative to cattle (50%; Rajakoski, 1960). The number of antral follicles in swamp buffaloes was reported to be only 20% of antral follicles in cattle (Ty et al., 1989). The number of non-atretic follicles (>1.7 mm) was between 1 and 5 (average 3) for buffaloes and 17 and 32 (average 22) for cattle (Ty et al., 1989).

The number of follicles recruited into follicular waves is relatively constant for individual buffaloes (Baruselli et al., 1997a; Neglia et al., 2007) and this is also observed in cattle (Boni et al., 1993). Given that the follicular response to ovarian superstimulation treatment appears to be related to the number of follicles available to be recruited at the start of treatment in cattle (Romero et al., 1991) it may be hypothesised that the heritability of non-atretic antral follicles may prove to be an important genetic parameter for selecting donor buffaloes in assisted reproduction programs. In this regard, the follicular response to ovarian superstimulation remained relatively constant for individual buffaloes (Misra et al., 1991).

The first follicular wave in buffaloes is apparent on day 3 of the oestrous cycle (day 0 = oestrus) (Baruselli et al., 1997a). The second follicular wave in 2-wave oestrous cycles is apparent later than in 3-wave estrous cycles (day 10.8 ± 1.1 and day 9.3 ± 1.2, respectively) and this is similar to cattle (Sirois and Fortune, 1988; Fortune, 1993). The growth and static phases of the first-wave dominant follicle in buffaloes differ in 2-wave and 3-wave oestrous cycles. The first-wave dominant follicle of a 2-wave oestrous cycle has a longer growth phase and a larger diameter than the first-wave dominant follicle of a 3-wave oestrous cycle (Baruselli et al., 1997a).

The maximum diameter of the first dominant follicle (13.3 ± 1.8 mm) in 3-wave oestrous cycles is larger than the maximum diameter of the second dominant follicle (11.1 ± 2.1 mm) (Baruselli et al., 1997a; Fig. 1). The second dominant follicle in 3-wave oestrous cycles in buffaloes occurs at the time of maximal circulating concentrations of progesterone which is the same as in cattle (Bergfelt et al., 1991; Sirois and Fortune, 1990; Adams et al., 1992; Bruke et al., 1994). This has led to the conclusion that the concentration of progesterone in circulation influences the size attainable by dominant follicles by regulating the secretion of LH (Savio et al., 1993). LH is important for steroidogenesis in dominant follicles and reduced secretion of LH depletes follicles of androgen substrate for aromatization, and this initiates atresia (Lucy et al., 1992).

It has been reported that there are no differences in the maximum diameter of the first dominant follicle and the ovulatory follicle for both 2-wave (15.1 ± 2.4 and 15.5 ± 1.6 mm, respectively) and 3-wave (13.3 ± 1.8 and 13.4 ± 1.3 mm, respectively) oestrous cycles in Murrah buffaloes (Baruselli et al., 1997a). However, Neglia et al. (2007) found that Mediterranean buffaloes had a maximum diameter of pre-ovulatory follicles which was greater than the diameter of dominant follicles of other waves in the cycle. In cattle, absolute size of both the first dominant and ovulatory follicles were smaller for 3-wave oestrous cycles (Bergfelt et al., 1991; Fortune, 1993). The follicular sizes observed in the study of Baruselli et al (1997a) were similar to follicular sizes reported for *B. taurus* cattle but greater than the follicular sizes previously reported for buffaloes (Manik et al., 1994) and Zebu cattle (Barros et al., 1993).

The deviation in growth of the largest (dominant follicle, DF) and second largest follicle (subordinate follicle, SF) of a follicular wave in buffaloes occurs at around 2.6 days after wave emergence (Gimenes et al., 2007) which is the same as in Zebu cattle (Sartorelli et al., 2005; Gimenes et al., 2008). At the time of deviation, the average size of the DF and SF in buffaloes (7.2 and 6.4 mm, respectively) are larger than in Zebu cattle (6.2 and 5.8 mm, respectively; Gimenes et al., 2007) but smaller than in *B. taurus* beef cattle (8.5 and 7.2 mm, respectively; Ginther et al., 1996). In Holstein dairy cattle, circulating concentrations of FSH show a decline at the time of follicle deviation or shortly thereafter and LH concentrations show a transient rise before follicle selection (Ginther et al., 2001). These interrelationships between FSH, LH and follicular dynamics have not been as clearly established in buffaloes (Gimenes et al., 2007; Fig. 2).

The emergence of the second and third follicular waves in buffaloes is apparent 2 or 3 days after the beginning of the static phase of the previous dominant follicle (Baruselli et al., 1997a) and this is similar to cattle (Ginther et al., 1989b; Fortune, 1993). This observation in buffaloes and cattle suggested that the dominant follicle looses its dominance at the beginning of the static phase even though morphological dominance is maintained (Fortune et al., 1991). Subordinate follicles of each follicular wave cease to grow after a few days. A new wave does not emerge whilst a dominant follicle is in the growing phase or at the very early stage of the static phase (Ginther et al., 1989b).

Wave-like patterns of follicular growth were observed in pre-pubertal Mediterranean Italian buffaloes at 5–9 months of age (Presicce et al., 2003). It is possible that in these young buffaloes the ovary not undergoing a follicular wave could also have a relatively large follicle suggesting that complete follicular dominance was yet to be established. Fluctuations in FSH and oestradiol were also observed to coincide with follicular development, although relationships between FSH and follicular wave emergence were less apparent than in mature animals (Presicce et al., 2003).

3.1.1. *Follicular response to exogenous hormones*

3.1.1.1. Induction of ovulation. Gimenes et al. (2007) reported that follicles in Murrah × Mediterranean buffalo heifers acquired the capacity to ovulate in response to exogenous LH when they reach a diameter of 8.5–10.0 mm. On the other hand follicles from multiparous Mediterranean cows with diameters ranging from 4.2 to 13.0 mm ovulated when treated with hCG or GnRH agonist (Campanile et al., 2007a, 2008a). In the latter studies, follicles ranging from 4.2 to 13.0 mm did not consistently ovulate after treatment with hCG or GnRH agonist suggesting that size does not necessarily reflect stage of follicle development or functionality.

In buffaloes undergoing regular oestrous cycles dominant follicles attain a size of 13–15 mm before ovulation (Baruselli et al., 1997a; Neglia et al., 2007) and this could be interpreted to suggest that, under normal conditions, follicles in buffaloes acquire the capacity to ovulate at around 5–8 mm but must undergo further development in order to secrete sufficient oestradiol for a positive feedback action to induce the pre-ovulatory LH surge. In support of this suggestion, it was found in Holstein that follicles of 8.5 mm had

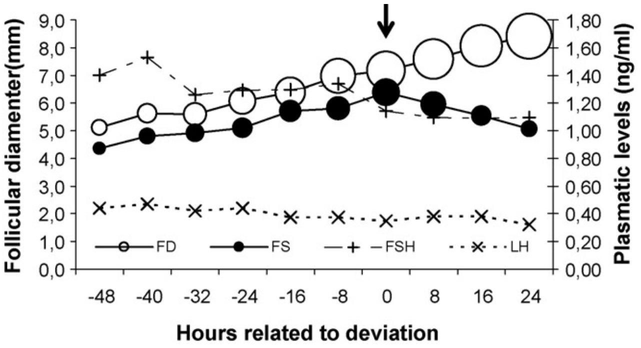

Fig. 2. Diameter (mean ± SEM) of the dominant follicle (○, FD) and largest subordinate follicle (●, FS), and profiles of FSH and LH normalised to the day of observed deviation in 10 Murrah buffalo heifers. The arrow indicates the start of deviation (Gimenes et al., 2007).

already undergone follicle deviation, but typically ovulated at diameters larger than 10.0 mm (Sartori et al., 2001). The authors hypothesised that this could be attributed to an increase in LH receptors with continued follicular growth.

3.1.1.2. Follicular superstimulation. Follicular superstimulation is most successful when the initiation of exogenous FSH treatment is timed to coincide with the endogenous wave-inducing surge of FSH and emergence of the second follicular wave. This is difficult to achieve in randomly cycling buffaloes because of the rather large variation in duration of the first follicular wave (Baruselli et al., 1997a). Ultrasonography can be used to monitor follicular waves but this is often not practical and an alternate approach is to use exogenous hormone treatments to control follicular wave emergence (Baruselli et al., 2002; Andrade et al., 2003; Baruselli and Carvalho, 2005). Buffaloes injected with oestradiol at the start of a 7-day progesterone treatment showed synchronous follicular wave emergence 4–5 days after the end of treatment (Bartolomeu et al., 2002). Furthermore, in a recent study (Gimenes et al., 2009) the interval between the oestradiol and progesterone treatments and the onset of follicular wave emergence was similar among buffaloes (4.5 ± 0.2 days), Holstein (4.4 ± 0.2 days) and Nelore (*B. indicus*; 4.2 ± 0.2 days) cattle. This treatment with FSH can be programmed to coincide with wave emergence after synchronisation.

A second requirement in follicular superstimulation is the ability to control the time of ovulation in order to implement fixed-time AI. The injection of GnRH or porcine LH 24 h after the last treatment with FSH results in predictable and synchronous ovulation in buffaloes which removes the need for oestrous detection for AI (Baruselli et al., 2002). In a superstimulation protocol that utilised a GnRH agonist to downregulate the anterior pituitary in buffaloes, the injection of porcine LH 24 h after the last injection of FSH is associated with greater ovulation rate, fertilisation and embryo recovery compared with injection of LH 12 h after the last FSH (Zicarelli et al., 2000). The injection of LH at 12 h induced follicle luteinisation without ovulation (Zicarelli et al., 2000) whilst injection of LH at 36 h after the last FSH induced ovulation in only a small proportion of buffaloes (Carvalho et al., 2002).

It can be concluded that the control of follicle emergence at the start of a superstimulation protocol is possible and the control of ovulation at the end of the protocol, can be achieved in multiple ovulation and embryo transfer programs in buffaloes.

3.2. Corpus luteum

The corpus luteum (CL) is an endocrine structure that develops after ovulation and has a fundamental role in the control of reproduction in mammals (Niswender and Nett, 1994; Niswender et al., 2000; Diaz et al., 2002). Regression of the CL in the absence of conception, and the associated discontinuation of progesterone secretion, is obligatory for the initiation of a new ovarian follicular wave and commencement of the next oestrous cycle. The weight of the CL in buffaloes at around the mid-luteal phase is between 1.0 and 1.5 g (El-Sheikh et al., 1967; Ghosh and Mondal, 2006) which is less than the weight of the CL in cattle. The relatively small size of the CL in buffaloes could explain, at least in part, reduced progesterone secretion and lower circulating concentrations of progesterone in buffaloes compared with cattle (Mondal and Prakash, 2004). Another feature of the CL in buffaloes is the tendency to be light-grey in colour rather than the typical orange colour in cattle, as buffaloes have reduced amounts of β-carotene in tissues (Ghosh and Mondal, 2006). In regular oestrous cycles, the CL in buffaloes secretes progesterone for around 15 days and maximal concentrations of progesterone in circulation range from 5 to 12 ng/ml between day 6 and day 16 of the oestrous cycle (Seren et al., 1995).

It has been proposed that the regression of the CL in buffaloes is a more extended process than in cattle based on a more gradual decline in circulating concentrations of progesterone in buffaloes (Fig. 3) (Danell, 1987; Avallone et al., 1988). In support of this suggestion, the RNA/DNA ratio of CL tissue in buffaloes did not change during the developing, developed and regressing phases (Ghosh and Mondal, 2006). On a tissue weight basis, total DNA and RNA did not differ between developing and regressing CL of buffalo (Ghosh and Mondal, 2006). Protein content was, however, lower on a tissue weight basis in the regressing CL (Ghosh and Mondal, 2006). In cattle, the RNA/DNA ratio was reported to decline during the regressing phase of the CL (Hafs and Armstrong, 1968; Mares et al., 1962).

Progesterone biosynthesis requires the transport of cholesterol to the inner mitochondrial membrane which

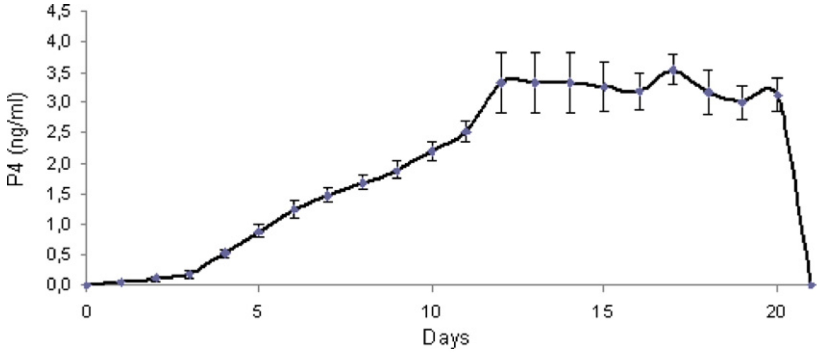

Fig. 3. Plasma progesterone (P4) concentrations during the oestrous cycle in buffaloes (Avallone et al., 1988).

is a process dependent on steroid acute regulatory protein (StAR) (Stocco, 2001). In an *in vitro* study, CL tissue from day 20 of the oestrous cycle had relatively high expression of StAR protein and this was associated with high circulating concentrations of progesterone (Kumar et al., 1991). This finding was interpreted to suggest that CL tissue on day 20 of the oestrous cycle in buffaloes was actively synthesising and secreting progesterone. In contrast, dairy cattle showed reduced expression of StAR protein towards the end of the oestrous cycle (Pescador et al., 1996). The contrasting observations for buffaloes and cattle could suggest that there are differences at the molecular level in mechanisms that regulate CL function.

In cattle, CL blood flow and vascularisation are closely correlated with progesterone synthesis and secretion (Stormshak et al., 1963; Kobayashi et al., 2001). Similar relationships between vascular density and progesterone synthesis were also observed in buffaloes at the mid- and late-luteal phases (Asahara et al., 1995; Zimmermann et al., 2001). In more recent studies in buffaloes, the expression of vascular endothelial growth factor (VEGF) by the CL changed during the oestrous cycle and was related to concentrations of progesterone in circulation, similar to cattle (Papa et al., 2007). Interestingly, receptors for VEGF are expressed on luteal cells suggesting that VEGF may influence steroidogenesis in the buffalo CL by as yet undetermined mechanisms (Kashida et al., 2001). Notwithstanding the need to understand in greater detail the interrelationships between VEGF and CL function, it can be concluded that CL function and angiogenesis are intrinsically related.

The first ovulation postpartum in buffaloes typically occurs between day 25 and day 70 (El-Wishy, 2007) and the first oestrus postpartum between day 44 and day 87. The first CL postpartum is usually characterised by a short luteal phase (6–13 days) and relatively low circulating concentrations of progesterone. Short oestrous cycles (<18 days) at the resumption of ovulation postpartum occur in around 25–45% of buffaloes (Perera et al., 1988; Khattab et al., 1990; Barkawi, 1993; Abdalla, 2003)

Other factors which can influence the function of the CL in buffaloes include season and the presence of the male. During the seasonal transitional period, which corresponds to mid-winter in the Mediterranean region, CL function as judged by CL size and concentrations of progesterone in circulation can be reduced by 5–50% (Campanile et al., 1992). A typical scenario for buffaloes entering the transition period to seasonal anoestrus is for a progressive decline in CL activity in successive cycles before entering anoestrus (Zicarelli et al., 1988b). The latter is more likely to occur in buffaloes 8 years and older. The proportion of females in a group that show reduced progesterone in cycles approaching the onset of anoestrus is reduced by the presence of a buffalo bull (Zicarelli et al., 1997).

3.3. *Corpus luteum and oestrus synchronisation*

Under conditions of normal oestrous cycles and normal CL function the response of buffaloes to oestrus synchronisation with $PGF_2\alpha$ is comparable to the response in cattle (Zicarelli et al., 1997). Accordingly, $PGF_2\alpha$ does not have a luteolytic action on the CL if administered either before days 4–5 or after day 16 of the cycle (Acosta et al., 2002; Neglia et al., 2008). A peculiar feature of the response in buffaloes to $PGF_2\alpha$ occurs when animals are transitioning to seasonal anoestrus. If a group of buffaloes has around 50% of females showing cyclic activity, then in response to $PGF_2\alpha$ approximately 30% became acyclic and fertility to AI at the synchronised oestrus is relatively low. If, on the other hand, the majority of females are showing cyclic activity at the time of treatment with $PGF_2\alpha$, then a high proportion remain cyclic and have relatively high fertility to AI at the synchronised oestrus (Zicarelli et al., 1988b). These findings would suggest that treatment with $PGF_2\alpha$ can advance anoestrus in transitional buffaloes that are already close to entering seasonal anoestrus.

It was reported that buffaloes with a relatively large ($\geq$1.5 cm) CL at the time of synchronisation with $PGF_2\alpha$ had higher fertility to AI compared with buffaloes that had a small ($\leq$1.1 cm) CL (Di Palo et al., 1990). It was also reported that buffaloes with a small CL and low concentrations of progesterone in circulation showed a reduced incidence of ovulation after synchronisation with $PGF_2\alpha$ (Brito et al., 2002). In a more recent study, the diameter of the CL was not directly related to progesterone concentrations in whey and, in the same study, pregnancy rate was greater for buffaloes that showed higher CL blood flow (Russo et al., 2009). The latter study demonstrated that the measurement of CL size does not provide an accurate indication of functionality and it is necessary to undertake colour Doppler ultrasonography in order to accurately ascertain CL status and the likely fertility response to synchronisation with $PGF_2\alpha$.

Buffaloes with decreased CL vascularisation also had a greater CL blood resistance index on day 25 after AI and this was associated with reduced foetal growth (Campanile, G., unpublished results).

4. Embryonic development and mortality

4.1. Embryonic development

Embryonic development in buffaloes is advanced by 12–24 h compared with embryonic development in cattle and this has been demonstrated by both *in vivo* (reviewed in Gasparrini, 2002) and *in vitro* studies (Neglia et al., 2001). The advanced embryonic development in buffaloes is associated with earlier entry of buffalo embryos into the uterus on around days 4–5 after oestrus (Karaivanov et al., 1987; Anwar and Ullah, 1998). In cattle, embryos are located in the uterus at around day 6 after oestrus. Compact morulae in buffaloes are observed from day 5 after oestrus and blastocysts typically from around day 6 (Neglia et al., 2001). Hatching of *in vivo* derived buffalo embryos was reported to occur from day 5 to day 7 in different studies (Gasparrini, 2002; Karaivanov et al., 1987; Drost and Elsden, 1985; Chantaraprateep et al., 1989; Zicarelli et al., 1993). The apparent variability in the time of hatching of blastocyst embryos could be related, at least in part, to the relatively diverse genotypes (riverine, swamp and crossbred buffaloes) studied.

Embryo elongation in buffaloes is thought to occur from around day 13, the pre-attachment phase from day 17 to day 24, and the transitory attachment phase is initiated around day 25 (Campanile and Neglia, 2007). Pregnancy-associated glycoprotein β is also reported to increase from day 25 in buffaloes (Karen et al., 2007).

As noted above, circulating progesterone reaches concentrations indicative of a functional CL ($\geq$1.5 ng/ml) around day 6 of the oestrous cycle in buffaloes (Avallone et al., 1988; Seren et al., 1995; Campanile et al., 2007a). Progesterone is essential for the conditioning of the uterine environment to support embryonic development. Given that embryos may enter the uterus at days 4–5 and hatching can occur from as early as day 5 it could be concluded that formation of the CL and initiation of progesterone secretion is vital in buffaloes. A delay in the secretion of significant progesterone, possibly by only 24 h can result in a uterine environment that is not supportive of newly hatched embryos in buffaloes. Given these scenarios, it is suggested that embryonic and CL development is more tightly coupled in buffaloes than in cattle (Psychoyos, 1973; Paria et al., 1993; Dey, 1996). In support of this suggestion, pregnancy rates in buffaloes appeared to be related to circulating concentrations of progesterone on day 5 (Campanile et al., 2007a).

4.2. Embryonic and foetal mortality

The windows for embryonic mortality in buffaloes occurs between day 15 and day 24 (early embryonic mortality) and days 25–45 (late embryonic mortality), with foetal mortality typically occurring from day 46 to day 90 (Vecchio et al., 2008). In buffaloes showing regular oestrous cycles, early embryonic mortality can result from inadequate secretion of progesterone to support embryonic development from features of the embryo that are independent of progesterone. In support of a role for progesterone in early embryonic mortality, buffaloes treated with $PGF_2\alpha$ at the time of AI had greater concentrations of progesterone in circulation on day 10 and day 20 compared with untreated buffaloes, and the former animals also had a 15% greater pregnancy rate (Neglia et al., 2008). Buffaloes transitioning to seasonal anoestrus also show an increase in progesterone above normal levels in response to treatment with a GnRH agonist on day 5 after AI. These animals have a higher pregnancy rate on day 40 compared to buffaloes that failed to show an increase in progesterone after treatment with a GnRH agonist (Campanile et al., 2007c, 2008a). In addition, for seasonally breeding herds, concentrations of progesterone are greater in oestrous cycles and early pregnancy during the breeding season compared with non-breeding season (Campanile et al., 1989).

In relation to late embryonic mortality (days 25–45) progesterone concentrations are typically normal on day 10 after AI but undergo a progressive decline thereafter (Fig. 4) (Russo et al., 2009). The decline in progesterone after day 10 may have implications that are related to the role of progesterone in the regulation of MUC-1 expres-

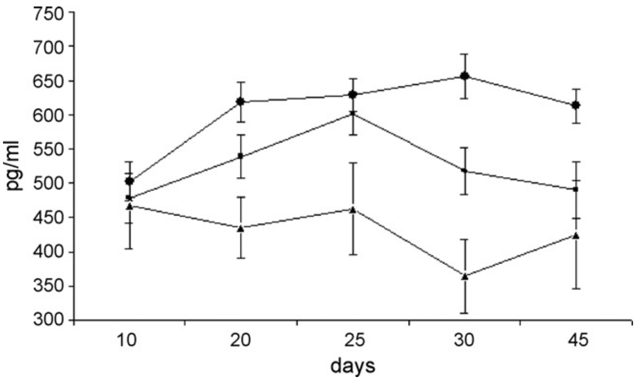

Fig. 4. Milk whey progesterone concentrations (pg/ml) on day 10, day 20, day 25, day 30, and day 45 after AI for pregnant buffaloes (●), buffaloes that underwent late embryonic mortality (▲) and buffaloes that underwent foetal mortality (■) (Russo et al., 2009).

sion in the uterine epithelium. The mucin glucoproteins are transmembrane receptors that are expressed on the apical surface of epithelium, including in buffaloes (Perucatti et al., 2006), and present a barrier to the developing blastocyst (Geisert and Malayer, 2000). Progesterone decreases MUC-1 expression which allows blastocyst attachment and the decline in progesterone from day 10 associated with late embryonic mortality is likely to result in continued MUC-1 expression and prevention of normal implantation processes.

The use of exogenous hormonal treatments in buffaloes designed to increase progesterone concentrations in circulation have produced variable outcomes in relation to the maintenance of embryonic development and embryonic attachment (Campanile and Neglia, 2007; Campanile et al., 2007a,b, 2008a,b; Kumar et al., 2003). It is possible that treatments have not been adequately synchronised to the chronology of embryonic events in buffaloes such as the rate of embryonic development and embryo transitioning into the uterus. As discussed above, embryonic development and movement to the uterus are more advanced in buffaloes compared with cattle. Some exogenous hormonal treatments in buffaloes have been based on information in cattle (Mann and Lamming, 2001; Mann, 2002) and it is suggested that the timing of hormonal treatments in buffaloes should be reviewed so as to better match endogenous embryonic events. For example, it is possible that treatments to reduce early embryonic mortality should be administered before day 5 after AI and treatments to reduce late embryonic mortality should be applied before day 25.

Buffaloes that undergo foetal mortality (day 46–90) can have normal progesterone on day 25 but usually decreased progesterone on days 30–45 after AI (Fig. 4) (Vecchio et al., 2008). Treatment with progesterone, GnRH agonist and hCG on day 25 maintained progesterone at concentrations similar to pregnant, untreated buffaloes (Campanile et al., 2008b) on day 30 and day 45 and reduced the incidence of late embryonic mortality, but there was no effect on the rate of foetal mortality on day 70 (Vecchio et al., 2008). The onset of progesterone secretion by placental tissue in buffaloes has not been determined whilst in cattle significant placental steroidogenesis occurs from around day 60 (Hoffmann and Schuler, 2002). If placental progesterone secretion is also a major source of progesterone from around day 50 in buffaloes then exogenous treatments which aim to maintain luteal progesterone secretion are unlikely to have a major impact on foetal development, as reported (Russo et al., 2009).

5. Conclusions

Buffaloes have a high capacity for adaptation and are an efficient production animal across broad climatic zones. In environments where there is no significant annual variation in photoperiod (equatorial zones) the major influence on reproduction is nutrition. Buffaloes become increasingly influenced by photoperiod with distance from the equator but nutrition remains important. This review has highlighted some fundamental differences in reproductive biology between buffaloes and cattle. In particular, embryos arrive at the uterus earlier in buffaloes which has led to a tighter coupling between early embryonic events and the onset of progesterone secretion in buffaloes compared with cattle. This has implications for the development of exogenous hormonal protocols to support embryonic development in buffaloes in conjunction with oestrus synchronisation and AI. The review has also identified a deficiency in basic understanding of embryonic attachment in buffaloes and it is suggested that this is an important frontier for discovery that will lead to the next major advance in technology to improve reproductive performance in buffaloes.

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Effects of clinical mastitis from calving to first service on reproductive performance in dual-purpose cows

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ABSTRACT

To determine the effects of clinical mastitis between calving and the first service on reproductive performance in dual-purpose cows under tropical conditions, the reproductive records of cows with clinical mastitis before first service ($n = 177$) were compared with the records of cows without mastitis ($n = 205$). Mastitic cows had more days to first service (136 days compare to 98 days, $P \leq 0.05$) and days to conception (187 days compare to 143 days, $P \leq 0.05$) than non-mastitic cows. These intervals were affected by parity, where the most severe effect of clinical mastitis was observed in primiparous cows, which showed a delay of 53 days to their first service when compared to control cows ($P \leq 0.05$). Timing of mastitis episode was also an important factor, affecting days to first service and days to conception in multiparous cows only when clinical mastitis occurred over 62 days after calving, while in primiparous cows these effects were observed with mastitis occurring at any time of postpartum period ($P \leq 0.05$). No differences were observed in the numbers of services by conception and first service conception percentage between groups. In conclusion, clinical mastitis before first service is a negative factor that increases the days to first service and the days to conception in dual-purpose cows under tropical conditions. Therefore, measures for mastitis prevention should be implemented to reduce the effect of this additional factor over the postpartum anestrus incidence in dual-purpose cows. Further research to understand the mechanism of the association between mastitis and reproduction should be granted.

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1. Introduction

Mastitis in cattle is considered the most important disease affecting milk production worldwide (DeGraves and Fetrow, 1993) and the second reason for poor reproductive efficiency as a result of discarded cows (Bascon and Young, 1998). In the last few years, mastitis has been reported to negatively affect the reproductive performance of dairy cows as evidence by increase in days to first service, increase of services per conception and the days to conception, alterations in inter-estrus interval, and a increase in abortions (Moore et al., 1991; Barker et al., 1998; Schrick et al., 2001; Santos et al., 2004; Ahmadzadeh et al., 2009). Such deleterious effects have been observed in cows with subclinical or clinical mastitis; however, when subclinical mastitis progressed to acute clinical cases, the negative impact in the reproductive performance is worse (Schrick et al., 2001). The timing of mastitis occurrence also appears

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to be important. If the disease occurred before the first service after calving, the numbers of days to first service and days not pregnant increase markedly; while when mastitis occurred between first service and pregnancy diagnosis, the days to conception and the services by conception increased (Barker et al., 1998; Loeffler et al., 1999; Schrick et al., 2001; Ahmadzadeh et al., 2009). The mechanisms behind the association between mastitis and reproduction are just starting to be studied and have been described elsewhere (Hockett et al., 2000, 2001; Suzuki et al., 2001; Hansen et al., 2004). In short, pro-inflammatory mediators and bacterial endotoxins that are released during the onset of mastitis are believed to affect the endocrine pathways (hypothalamus-pituitary-ovary-uterus axis) altering the reproductive performance of cows, causing anestrus, anovulation, infertility, and other problems related to hormone imbalances.

The majority of the reports about this relationship (mastitis and reproduction) thus far have been performed in temperate countries with high-milk production, pure dairy breed (*Bos taurus*) cows (Barker et al., 1998; Santos et al., 2004; Ahmadzadeh et al., 2009). In contrast, in most tropical areas, the dairy industry relies on dual-purpose crossbreed cows to satisfy their own milk production needs. These dual-purpose cows are a cross between different European (*B. taurus*) and Zebu (*Bos indicus*) breeds, principally Holsteins or Brown Swiss and Brahman, respectively (Rubio et al., 1999). In general, these breeds are characterized by having less milk production, poorer genetic merit, and less resilience to the tropical environment. The reproductive efficiency of these cows is characterized by a long postpartum anestrous period and is affected by several factors such as suckling (Soto et al., 1997a), season (Rubio et al., 1999), bull biostimulation (Soto et al., 1997b), and parity, among others (Cristiani et al., 1993). However, the effect of mastitis on reproductive performance in dual-purpose cows is in general unknown. Therefore, the purpose of this study was to determine the effects of clinical mastitis, occurring between calving and the first service, on reproductive performance in dual-purpose dairy cows, under tropical conditions. We hypothesized that even though dual-purpose crossbreed cows have lesser milk production and a smaller mammary gland, clinical mastitis can still have a negative impact on reproduction as occurs in pure high-milk production dairy cows, especially during the critical period after calving and before the first service. Determining the significance of this effect of clinical mastitis on reproduction is important in dual-purpose cows because these animals serve as the main source of animal protein (milk and meat) in many countries.

2. Materials and methods

This was a 1-year-long retrospective study analyzing the reproductive records of a large herd of dual-purpose crossbred cows from a commercial farm. This commercial farm was particularly chosen as they possess a system with improved management, characterized by technology application such as computerized reproductive and health records, artificial insemination, and have genetic, nutritional, and preventive medicine standard operating procedures. This farm also has 24-h on-location veterinarians year round. The farm is located in Machiques of Perija County, Zulia State, Venezuela. This area is a subhumid tropical forest with a mean daily temperature of 28.3 °C, 1950 mm/year of rainfall, and a relative humidity of 60–70%. The farm had, on average, 8000 cattle during the study period, of which 4418 were dual-purpose cows (3534 lactating cows and 884 non-lactating cows). The animals grazed in pasture consisting of German grass (*Echinochloa polystachya*) and Braquiaria grass (*Brachiaria humidicola*), with access to a commercial mineral mix *ad libitum*, as a dietary supplement.

Manual milking was performed twice at day after a short sucking by the cow's own calf to stimulate milk release. During the milking, the calf remained bound to a cow's forelimb. After milking, the cows were again suckled by their own calves to remove residual milk from the udder. The average milk production per cow was 6.5 kg/day, with a 270-day lactation period. Clinical mastitis was defined as abnormal milk (presence of discoloration, water-like consistency, clots, flakes, and/or blood) and inflammation of one or more quarters of the udder. Cows with clinical mastitis were identified by milking personnel during the milking routine, whom then notified the veterinarian in-charge. The veterinarian would then confirm the diagnosis and determine which cows required treatment, and maintained records of each case. Only cows with one case of clinical mastitis between calving and first service, without other concomitant diseases, were used in this study. No samples of foremilk from quarters of cows with clinical mastitis were analyzed for bacteriology; neither the California Mastitis Test nor a Somatic Cell Count was performed.

Estrus was detected by visual observation of cow estrus behavior over a period of 1 h each morning (6:00–7:00 am) and afternoon (6:00–7:00 pm), and through the use of non-entry bulls (1 bull to 25 cows) for biostimulation. All cows were inseminated after detection of the first estrus by one technician, in accordance with the international am–pm rule. If cows did not return to estrus, an experienced veterinarian performed pregnancy diagnosis by rectal examination 45 days post-insemination service. The farm maintained computerized and paper records of the reproductive performance of each cow, including day of calving, day of first service, and number of services. The reproductive records of cows with clinical mastitis before first service ($n = 177$, 42 primiparous and 135 multiparous) were compared with the reproductive records of cows without clinical mastitis ($n = 205$, 53 primiparous and 152 multiparous). Data were balanced by parity and calving season.

The group of cows with clinical mastitis was further categorized into four groups based on the time in which clinical mastitis occurred postpartum. Group 1 (G_1), had clinical mastitis in the first 6 days after calving; Group 2 (G_2), had clinical mastitis between 7 and 29 days after calving; Group 3 (G_3), had clinical mastitis between 30 and 62 days after calving; and Group 4 (G_4), had clinical mastitis over 62 days after calving.

Data were analyzed using a commercial statistical computer software program (SAS, 2001). The comparison between control and cows with mastiti, and between

Table 1
Effect of the timing of clinical mastitis occurrence (TCMO) before the first service on the reproductive performance of dual-purpose cows.

Group	n	Days to 1st service	Days to conception	Services per conception	FSCR[*] (%)
Control	205	$98.53 \pm 4.52^{A,a}$	$143.95 \pm 7.45^{A,a}$	$2.21 \pm 0.16^{A,a}$	$56.10^{A,a}$
Mastitis	177	136.31 ± 5.22^{B}	187.21 ± 8.60^{B}	2.35 ± 0.18^{A}	49.72^{A}
TCMO groups[&]					
G$_1$	44	101.38 ± 8.28^{a}	158.63 ± 14.00^{a}	2.84 ± 0.30^{a}	38.64^{a}
G$_2$	38	106.16 ± 8.90^{a}	142.71 ± 15.06^{a}	2.23 ± 0.32^{a}	47.37^{a}
G$_3$	48	97.08 ± 7.92^{a}	125.35 ± 13.40^{a}	1.83 ± 0.28^{a}	62.50^{a}
G$_4$	47	175.70 ± 8.01^{b}	225.59 ± 13.55^{b}	2.25 ± 0.29^{a}	48.94^{a}

Values with different superscripts within columns differ at $P \le 0.05$. A, B = Comparison between control and clinical mastitis cows. a, b = Comparison between control and TCMO groups.

[*] FSCR: First service conception rate.

[&] G$_1$: clinical mastitis <7 days postpartum. G$_2$: clinical mastitis between 7 and 29 days postpartum. G$_3$: clinical mastitis between 30 and 62 days postpartum. G$_4$: clinical mastitis over 62 days postpartum.

the different time of clinical mastitis occurrence groups (G1–G4), with regards to days to first service, days to conception, and service per conception was performed using analysis of variance. The variables representing parity and season were included in each model to control for potential confounding. The correlation between postpartum days of mastitis episode and days to first service, in addition to days to conception was also determined. The chi-square test was used to compare proportions including mastitis by season (rainy compare to dry), as well as the probability of conception at first service and pregnancy at 200 days between control and mastitis cows. Differences were considered significant at $P < 0.05$.

3. Results

From a total population of 3534 cows milked during the 1-year study period, 177 showed clinical mastitis before first service (5.0%). The presence of clinical mastitis was highly influenced by season, as 75.1% ($n = 133$) of the clinical mastitis cases occurred during the rainy season (from May to November), compared with 24.9% ($n = 44$) in the dry season (from December to April) ($P \le 0.05$). However, the season did not have a direct effect on the reproductive parameters measured in this study.

3.1. Days to first service

The results obtained in the analysis of days to first service are summarized in Table 1. A difference ($P \le 0.05$) of approximately 38 days was observed between control cows and cows with clinical mastitis. Parity had an effect ($P \le 0.05$) on the interval to first service, with primiparous control cows having 112.11 ± 7.72 days to first service in comparison with multiparous control cows with 84.94 ± 4.65 days. In cows with mastitis, the difference in days to first service between primiparous and multiparous cows increased to 59 days ($P \le 0.05$) (Table 2). In comparison with control cows, clinical mastitis increased the days to first service in both primiparous and multiparous cows, but had the greater impact on primiparous cows by delaying the first service in 54 days in comparison with multiparous cows, where the delay was only 22 days (Table 2). Calving season (dry compare to rainy season) did not affect the days to the first service (117.28 ± 5.45 and 117.56 ± 3.94 days, respectively, $P > 0.05$), additionally, no interaction effect of season by group on days to first service days was observed.

3.2. Days to conception

Cows with mastitis had 43 more days open than the control cows ($P \le 0.05$) (Table 1). When the effect of parity for this variable was analyzed, non-mastitic primiparous cows had 36 more days not pregnant than non-mastitic multiparous cows ($P \le 0.05$). This difference increased to 86 days when clinical mastitis was taken into account ($P \le 0.05$) (Table 2). Calving season, dry compare to rainy season, did not affect the days to conception (166.76 ± 8.98 compare to 164.40 ± 6.49, respectively, $P > 0.05$). No interaction effect of season by group on days to conception was observed.

3.3. First service conception rate and services per conception

No differences ($P > 0.05$) were observed for first service conception rate and services per conception between the controls and cows with mastitis (Table 1). Neither parity (Table 2) nor the calving season (53.45 compare to 53.01% and 2.29 ± 0.19 compare to 2.28 ± 0.14 s/c to dry and rainy

Table 2
Effect of clinical mastitis before the first service, adjusted by parity, on the reproductive performance in dual-purpose cows.

Group	Days to 1st service		Days to conception		Services per conception		FSCR[*] (%)	
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous
Control	112.11 ± 7.72^{b}	84.94 ± 4.65^{a}	161.82 ± 12.72^{e}	126.08 ± 7.66^{d}	2.20 ± 0.27	2.23 ± 0.16	60.38	54.61
Mastitis	165.66 ± 8.75^{c}	106.97 ± 5.24^{b}	230.16 ± 14.42^{f}	144.26 ± 8.63^{d}	2.47 ± 0.31	2.22 ± 0.18	42.86	51.85

Values within columns with different superscripts differ at $P \le 0.05$.

[*] First service conception rate.

Table 3
Effect of the timing of clinical mastitis occurrence before the first service, adjusted by parity, on days to first service and conception in dual-purpose cows.

Group	Days to 1st service		Days to conception	
	Primiparous	Multiparous	Primiparous	Multiparous
Control	112.11 ± 7.72^{a}	84.94 ± 4.65^{a}	161.82 ± 12.72^{a}	126.08 ± 7.66^{a}
G_1	145.91 ± 14.95^{b}	84.68 ± 9.15^{a}	226.50 ± 25.89^{bc}	133.18 ± 15.85^{a}
G_2	172.00 ± 16.37^{bc}	82.64 ± 9.78^{a}	216.90 ± 28.36^{bc}	126.21 ± 16.95^{a}
G_3	134.85 ± 19.57^{b}	90.63 ± 8.08^{a}	184.14 ± 33.90^{b}	115.31 ± 14.00^{a}
G_4	195.76 ± 14.36^{c}	168.02 ± 8.88^{b}	267.07 ± 24.87^{c}	209.73 ± 15.38^{b}

G_1: clinical mastitis <7 days postpartum. G_2: clinical mastitis between 7 and 29 days postpartum. G_3: clinical mastitis between 30 and 62 days postpartum. G_4: clinical mastitis over 62 days postpartum. Values with different superscripts within columns differ at $P \leq 0.05$.

season, respectively) affected the first service conception rate or the number of services per conception ($P > 0.05$). Additionally, overall pregnancy rate at 200 days postpartum was different ($P \leq 0.05$) between control cows and those with mastitis (81.46% compare to 72.32%, respectively).

3.4. *Effects of mastitis time of appearance on reproductive performance*

When the clinical cases were categorized accordingly to the time of appearance postpartum: $G_1 = 44$ cases, $G_2 = 38$ cases, $G_3 = 48$ cases and $G_4 = 47$ cases, no difference was observed in the days to first service among the control and G_1, G_2 and G_3 groups. However, a very large difference (77 days) was observed between the G_4 group and the control and G_1, G_2 and G_3 groups ($P \leq 0.05$). A positive correlation was observed between the postpartum days to mastitis episode and days to first service, $r = 0.523$ ($P \leq 0.05$). Similarly, the increase in days to conception was only significant in cows with clinical mastitis, occurring over 62 days postpartum, group G_4 (Table 1), and a positive correlation between the postpartum days to mastitis episode and days to conception was also observed, $r = 0.373$ ($P \leq 0.05$). However, when parity was included in the analysis, this effect was only observed in multiparous cows; while in primiparous cows, mastitis increased the days to first service and conception regardless of the time of mastitis occurrence in the postpartum period (Table 3). Timing of clinical mastitis did not affect the first service conception rate or services per conception (Table 1). Nevertheless, these results should be taken with caution given the limited number of cows according to parity, per group (G_1: 12 primiparous, 32 multiparous; G_2: 10 primiparous, 28 multiparous; G_3: 7 primiparous, 41 multiparous; and G_4: 13 primiparous, 34 multiparous). The interval between mastitis episode and first service was 110.92 ± 8.72 and 63.25 ± 4.86 days to primiparous and multiparous cows, respectively ($P \leq 0.05$).

4. Discussion

This study compared the reproductive performance in dual-purpose cows with or without clinical mastitis, before first service. The main objective was to investigate the possible negative role of clinical mastitis on reproduction in crossbreed cows with typical milk production under tropical conditions, as compared to reports in cows with high genetic merit for milk production in temperate countries (Barker et al., 1998; Loeffler et al., 1999; Schrick et al., 2001; Ahmadzadeh et al., 2009). The results from this study showed that clinical mastitis occurring before first service postpartum significantly increased the days to first service (38 days), and consequently increased days to conception (43 days).

Parity had a significant effect on days to first service and days to conception, an effect that was significantly increased when clinical mastitis occurred. Therefore, based on results from the present study, mastitis might have a major impact in primiparous cows, delaying the first service up to 53 days and conception up to 68 days.

When the timing of mastitis occurrence was considered in the analysis, the negative effect on days to first service and conception was most pronounced in cows that had the clinical mastitis case after 62 days postpartum (G_4). This is immediately following the voluntary waiting period when cows become eligible to service as soon as the first estrus is detected. The results from the present study suggest that multiparous cows having clinical mastitis after 62 days postpartum may add 83 days to their first service and in consequence a similar increase in days to conception. In primiparous cows, the increase in days to first service and conception was observed regardless of the timing of mastitis during the postpartum period (Table 3).

These results suggest that the effects of clinical mastitis on the reproductive performance (probably by affecting the hypothalamus-pituitary-ovary axis) in primiparous cows could be more severe possibly due to certain characteristics that first calving cows possess, such as: lesser energy balance, poor dry matter intake, greatest loss in body condition, lesser concentrations of glucose, and insulin as well as insulin-like growth factor-I (IGF-I) (Lucy et al., 1992; Taylor et al., 2003). Although, there are no published studies about the relationship between mastitis and endocrine alterations in primiparous cows, in the present study it was observed in primiparous cows days between a mastitis episode and first service was greater than multiparous cows, and this could be an indication of significant hormonal alterations delaying onset of ovarian activity postpartum. Furthers studies should be focused in this regards.

The presence of clinical mastitis before first service apparently was not one of the factors compromising the first service conception rate of these dual-purpose cows, and was only associated with an increase in days to first service and in consequence delaying the time of conception. Results from the present study are consistent with the observations reported by Barker et al. (1998), who indicated that clinical mastitis before first service increased the days to first service and conception, but did not affect fertility. The absence of effects on fertility observed in the present study could be a consequence of the longer interval between mastitis episodes and first service. Loeffler et al. (1999) observed that likelihood of pregnancy was greater as interval between mastitis episode and services increased. However, Santos et al. (2004), observed that clinical mastitis before service decreased the pregnancy rate, and in the present study, cows with mastitis had a lesser pregnancy rate at 200 days postpartum.

In conclusion, to our knowledge this study is the first report of the harmful impact of clinical mastitis occurring before the first service postpartum on the reproductive performance in dual-purpose cows under tropical conditions, mainly increasing the days to first service. This study also reports that clinical mastitis had a greater negative impact on reproductive performance in primiparous cows than multiparous cows. The negative impact of mastitis on reproduction underlines the importance of avoiding mastitis during the early postpartum period, highlighting the importance of mastitis preventive measures as the rule and not the exception. Further investigation is necessary to better understand the mechanisms by which mastitis affects reproductive performance in dual-purpose cows and to design better strategies to avoid these negative effects.

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Bio-economic evaluation of the use of sexed semen at different conception rates and herd sizes in Holstein populations

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ABSTRACT

A stochastic bio-economic model was used to determine the effects of applying artificial insemination (AI) with conventional or sexed semen at different conception rates and herd sizes on the genetic progress of productive trait and net profit over a 15-year period. The genetic means of the cattle population was predicted to increase over the years and the rate of genetic progress in the population program using sexed semen was significantly greater than the rate of progress in the population using conventional semen. The theoretical genetic gain was greater than the realized genetic gain for milk yield for all the AI scenarios. In addition, the genetic means for milk yield in the expanding herds were greater than in the fixed sized herds. The trend in net profit generated by using AI strategies with conventional semen over the time horizon was greater when herds were expanding ($P < 0.01$). The trend in net profit for fixed sized herds was generally negative, however, expanding herds experienced positive net profits over the years. Also, the net profit of using AI strategies with sexed semen was greater when dairy herds were expanding. On the other hand, the net profit of using AI with sexed semen was greater than that of AI with conventional semen at the end of the time horizon. The results of sensitivity analyses indicated that a 20% increase or decrease in milk sales income and feed costs have significantly modified the net profit of AI strategies using either sexed or conventional semen.

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1. Introduction

Reproduction on a dairy farm is a major determinant of performance and profitability. Without reproduction, a cow or heifer cannot start her lactation and produce milk. Reproduction also produces new dairy calves that can serve as replacement animals. Most genetic traits can be manipulated effectively in breeding programs, but gender is a special case for which animal breeders simply have to accept the probability that 51% of each bovine conception will result in a bull calf. Reproductive technology for cattle has made significant progress over the past 50 years. This is a continuum which began with the development and widespread use of artificial insemination (AI). The use of AI was greatly enhanced with cryopreservation of semen and the ability to successfully synchronize estrus and or ovulation. The beef and dairy industry has now focused on identifying elite sires and their widespread use.

The possibility of gender pre-selection has always generated great interest among livestock producers and the cattle industry. Sexed semen could contribute to increasing the profitability of both the dairy and beef industries through the production of offspring of the desired gender offspring production. The best examples would be the production of females for dairy or males for meat production. Other applications would allow cattle breeders and AI semen companies to test elite bulls on a small number of females (Hohenboken, 1999).

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The predicted impacts of sexed semen on genetic progress vary widely, as do projections of its impact on the profitability of dairy farmers. Van Vleck and Everett (1976) estimated that producers could afford to pay as much as \$15.67 per unit for sexed semen, assuming an average cost of \$6.00 for unsexed semen. Later, Van Vleck (1981) revised this estimate to \$19.00 per unit of sexed semen and concluded that the rate of genetic progress in dairy cattle could increase by 15% if this technology was adopted. However, later studies have predicted a much more modest improvement in genetic progress. Baker et al. (1990) suggested that the ability to alter the sex ratio of the offspring of elite sires and cows would have a very minor impact on the rate of genetic progress, but would have a major effect on the efficiency of farming systems, particularly in situations where male dairy calves have minimal (or perhaps negative) economic value.

The primary objective of this study was to evaluate the effect of applying sexed semen on the genetic trend for milk yield in simulated Holstein dairy herds. The second objective was a bio-economic evaluation of the use of sexed semen at different conception rates in comparison with conventional semen in simulated Holstein dairy herds.

2. Materials and methods

2.1. Model description

Stochastic dynamic simulation was demonstrated to be useful for measuring the performance of dairy herds. The objective of using a dynamic model for this study was to mimic the biology and management of a herd of individual youngstock and cows over time, with emphasis on reproduction and life time performance. The inclusion of stochastic functions generates variability in the performance of animals. This results in realistic variability in herd performance. Animals move forward in time from month to month. Each cow and heifer is described by a state. The states are characterized by actual milk production, weight, and reproductive status. The state of the individual animal is updated, and the production and feed consumption of the herd are calculated.

Dairy Herd Sim 1.0 simulation model (Honarvar, 2009) written in Visual Basic version 6.0 with appropriate changes to this model for evaluating the objectives of this study. Default input parameters used in the model are given in Table 1. The feed intake and body weight functions are deterministic. The milk production functions are stochastic and depend on milk producing ability, age, lactation number, day of lactation, and reproductive status. Inputs and outputs of animals from the herd are also estimated. The drawing of random numbers using relevant probability distributions triggers discrete events like pregnancy, and culling. The production and the development within the herd are thus determined indirectly by the simulation of production and change in status of the individual animals. The initial herd consists of a list of heifers in the herd described by their status variables. The reproductive process is viewed as a sequence of the events involving calving, estrous detection, service, conception, and abortion or subsequent calving. Cows in the model move from one event to the next, dependent on the outcome of previous events. When the model reaches the day on which a previously scheduled event occurs, the outcome of the event is determined and the next event for that animal is scheduled. The maximum herd size in the model is set by the user. Two sizes of dairy herds were used in the current study: fixed sized herds and expanding herds. Gender of the calf was determined by a $U(0, 1)$ random variable. Bull calves make up 52% of all calves born (Ghavi Hossein-Zadeh et al., 2008). The remainder was heifer calves. If sexed semen was used, and the cow became pregnant, the probability was set to 0.9 for the favored gender (female gender). Incidence of natural twinning was set at 1.10%, 3.20%, 4.22% and 4.50% of all calvings for first, second, third, and later lactations cows, respectively (Ghavi Hossein-Zadeh et al., 2009). The calving event triggered the determination of the calf data, and when the cow commenced estrous cycles the first ovulation. Scheduling of the second and later ovulations depended on whether the cow was served or not. The default probability for estrous detection (the estrous detection efficiency) was set at 0.50. But it was assumed that estrous detection efficiency was set at 0.9 when the estrous cycles of the cows and heifers were synchronized in the herds. Cows were eligible to be served following a detected estrus after a voluntary waiting period of 45 days after calving. Cows which were open more than 400 days were not served, but kept in the herd until they were culled. It was assumed that embryonic/foetal mortality before 42 days after service was included in the probability of conception. Foetal mortality after 42 days and up to 260 days was assumed to be an abortion and was scheduled separately. The probability of abortion for each month of pregnancy was calculated from current database of Holstein dairy cows in Iran. It was assumed that the odds ratio for abortion rate was 1.22 for single births versus twins (Ghavi Hossein-Zadeh et al., 2008). In addition, the odds ratio of stillbirth was assumed as 7.58 for twin births versus singletons (Ghavi Hossein-Zadeh et al., 2008). Served cows that do not return to estrus were diagnosed for pregnancy at 42 days after service. Pregnant cows were dried off at 220 days of gestation, regardless of pregnancy diagnosis result. Therefore, the dry period lasted for an average of

Table 1
Default input variable values used in the model.

Parameter	Value
Number of replications	40
Breeding period, year	7
Simulation period, year	21
Size of the herd	5000
Birth weight, kg	42
Mature live weight, kg	600
Heat detection rate, %	50
Synchronization rate, %	90
Conception rate (conventional semen), %	40
Conception rate (sexed semen), %	20, 30
Voluntary waiting period, day	45
Calf mortality, % of calves born	5
Cow death rate from weaning to first calving, %	2
Mean gestation length, day	280
Age at first freshening, month	24
Genetic variance of milk, kg^2	292,852
Residual variance of milk, kg^2	757,000

60 days. The average conception rate of the conventional semen used was assumed to be 40%. Stillbirth was defined as the loss of a calf from day 260 until the end of normal gestation period. Wood's function (Wood, 1967) was used to describe milk production. Parameters for functions for milk production were estimated from the test day records collected from the milk production records of dairy herds collected by Animal Breeding Center of Iran using Proc Nlin in SAS (2002).

Records of milk production were generated by assuming an infinitesimal additive genetic model as $y_i = \mu + a_i + e_i$, where y_i = phenotypic lactation milk record of the animal i; μ = population mean; a_i = additive genetic effect of the animal i; and e_i = random residual effect. The breeding value of animal i, a_i, was generated by $a_i = z\sigma_a$. If parents were unknown, where z is an independent random normal deviate and σ_a is the assumed additive genetic standard deviation, or was generated by $a_i = 0.5(a_{i(s)} + a_{i(d)}) + M$. If parents were known, where $a_{i(s)}$ and $a_{i(d)}$ are true breeding values of sire and dam of animal i and M is a deviation due to random Mendelian sampling, which was generated as $M = z(0.5(1-\bar{F}))^{(1/2)}\sigma_a$, where $\bar{F}$ was the average inbreeding of the parents, and $0.5(1-\bar{F})$ accounted for the reduction of genetic variation due to inbreeding. Also the residual effect, e_i, was generated as $e_i = z\sigma_e$, where σ_e was the assumed residual standard deviation.

Body weight was calculated as a function of age, lactation, and pregnancy following Korver et al. (1985):

$$BW_{t_a t_l t_p} = A\left[1-\left(1-\frac{y_0}{A}\right)\exp(-kt_a)\right]^3 + p_1 t_1 \exp\frac{(1-(t_1/p_2))}{p_2} + p_3^3 t_{pc}^3$$

where $BW_{t_a t_l t_p}$ is the cow's body weight (kg); t_a is her age in days; t_l is the number of days in lactation; t_p is the number of days pregnant; A is mature live weight (kg); y_0 is birth weight (kg); k is a growth rate parameter; p_1 is the maximum decrease of live weight during the lactation (kg); p_2 is the number of days during the lactation with the minimum live weight (kg); p_3 is a pregnancy parameter; $t_{pc} = t_p - 50$ when $t_p - 50 > 0$, otherwise $t_{pc} = 0$.

Interval from calving to first service, conception, and calving were calculated as the number of days between calving and first service, conception, and previous calving, respectively. Pregnancy rate was a function of conception rate and estrous detection rate. The estrous cycle was assumed to the 18–24 days. Conception rate, estrus detection rate, and estrus cycle period were sampled from a random uniform distribution between 0 and 1. Different equations adapted from NRC (2001) were used to compute dry matter intake, energy and protein requirements for maintenance, pregnancy, milk, and growth per animal.

The theoretical annual rate of genetic gain for milk yield in a herd using conventional AI was 150 kg/year. The realized rate of genetic gain was assumed to be a third of the theoretical gain, i.e., 50 kg/year (Van Vleck, 1977). Therefore, cows and heifers were bred to sires with milk genetic predicted difference of 50 and 150 kg/year. But, the genetic trend for milk in the cow population in which sexed semen used was assumed to be 9% greater than that using conventional semen (Hohenboken, 1999).

Table 2
Summary statistics of economic variables for dairy herds in this study.

Variable	Value
Milk sales, $/kg	0.42
Meat price, $/kg	2.7
Heifer price, $	4000
Carcass weight price, $	1.7
Price of male calf at birth, $	300
Price of female calf at birth, $	600
Veterinary costs, $/cow/year	163
Synchronization cost, $	5
Conventional semen, $	20
Sexed semen, $	40

2.2. Bio-economic evaluation

Summary statistics for some economic variables are shown in Table 2. Economic information was derived from different dairy farms to obtain realistic values for the Ira-

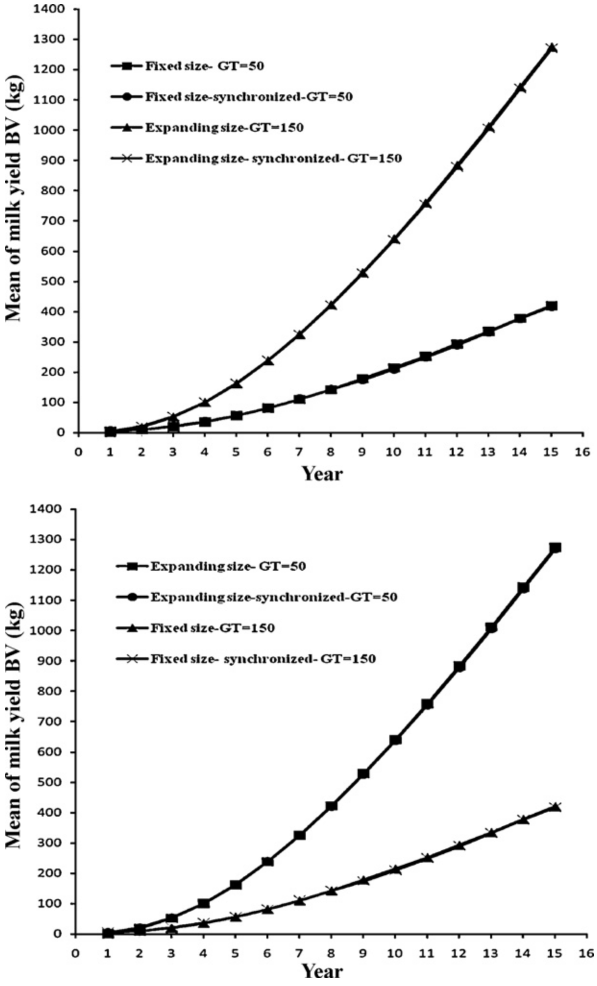

Fig. 1. The genetic mean (GT) of milk production for breeding cows and heifers with milk genetic trends of 50 and 150 kg/year to the cows and heifers in the fixed sized and expanding herds.

nian dairy industry. The 15 milk production classes were calculated as described by Van Arendonk (1985) with the lowest class equal to 70% of the average daily milk production and the highest class equal to 130%. Typically, annual veterinary costs per cow were estimated for an average first lactation cow and increased 5% for subsequent lactations. Groenendaal et al. (2004) assign 33% of these costs to the first month, 11% to the second and third months, and 5% to the later months of each lactation.

The present economic study started with the assumption that cows in the twin group had a naturally occurring twin pregnancy and therefore had no embryo transplantation. Also, only the effects of twinning were considered; ignored were causes and risk factors and direct influences of twin births on feed consumption and on diseases other than reproductive problems of the dam. The additional income was exclusively based on the returns from the calves. The value of the calf was based on weight, gender, breed, mortality, and the price per kilogram of body weight. In the present study, the birth weight of twin calves was assumed to be 8 kg lighter than for singles (Ghavi Hossein-Zadeh, 2010).

The net return (profit) for the system was calculated as the sum of daily income from the milk, male calves, culled cows, surplus heifers, and other products (total income) minus total costs including feed, veterinary and health, livestock supplies, real estate and equipment repairs, trucking and miscellaneous. To test the sensitivity of the model, milk payment, meat and heifer prices, feed and non-feed costs were varied by ±20%.

3. Results and discussion

The genetic means for milk yield for different herd sizes, using AI programs, either with sexed or unsexed semen (with or without estrus synchronization) are presented in Fig. 1. The results of this study indicated that the genetic means of AI population were increased over the years, because cows and heifers were bred to sires with milk genetic trends of 50 and 150 kg/year, respectively (Fig. 1). The rate of genetic progress in the AI program with sexed semen was significantly greater than the rate of progress in AI population with conventional semen (Tables 3 and 4). This would be explained by the greater genetic trend of sexed semen than that of conventional semen. On the other hand, as expected, the theoretical genetic gain was greater than the realized genetic gain for milk yield in the AI scenarios. In addition, the genetic means for milk yield in the expanding herds were greater than in the fixed sized herds, using different scenarios in the dairy herds. The possible reason for the greater rate of genetic progress in expanding herds was due to the larger size of expanding herds than the size of static herds. Expanding herds probably experienced lower inbreeding rates than fixed sized herds. Therefore, there were greater possibilities for the use of better genetic material and the better expression of the genetic potential of dairy cows for production traits in the expanding herds.

The net profit of using different reproductive and management strategies in the dairy herds are shown in Tables 5–7. In general, the net profit from the AI strategies was positive at the end of time horizon. The trend of net profit of using conventional semen over the time horizon was greater when the size of the herds was under expansion ($P < 0.01$). This increase was due to the genetic trend for milk yield and the increase in the milk production over the years. Also, the trend of net profit over the time horizon for AI strategies with sexed semen was greater when the size of herd was fixed and the estrous cycles of the cows and heifers were synchronized in the herds. But the trend of net profit for fixed sized herds was generally negative, however, expanding herds experienced the positive net profits over the years. This was likely due to the greater genetic trend of milk yield in expanding herds than in fixed sized herds over the years. On the other hand, the net profit of using sexed semen was greater than that of conventional semen at the end of the time horizon. There were significant year to year variations in the net profit of using AI with sexed semen (Tables 6 and 7). These variations were likely due to the significant difference in the number of surplus heifers from one year to another and consequent incomes from surplus heifer sales across the years. In other words, the greater the difference in the number of surplus heifers from year to year, the higher the year to year difference of the net profit.

A key outcome is that most performance variables, such as number of AI services, number of voluntary culls, and number of surplus dairy heifers, are sensitive to conception rate differences between AI strategies. In general, the percentage of cows finally pregnant is lower where the conception rate is poorer. As expected, total number of services required increased as the conception rate decreased. Synchronized service had minimal effect on final pregnancy rate, in part because submission rate was 90% for all scenarios. However, synchronization improved the mean calving date because the start of breeding coincided with synchronization. In practice, non-pregnant cows are the preferred candidates for culling. The total number of cows culled is constant in herds of fixed size and is determined by the number of cows surplus to the required number of lactating cows. Benefits were shown for estrous synchronization as a companion technology because synchronization would reduce the number of days that cows and heifers need to be fed per insemination. In addition, the sex-controlled scenarios resulted in many more female calves than sex-uncontrolled scenarios. This allows the farmer to select animals with the best genetic potential as dairy replacement heifers, therefore, the rate of genetic gain increased in the dairy herd. Concurrent use of estrous synchronization in AI programs with conventional semen resulted in more net profit than non-synchronized programs (on average from 4 to 13 $/year). This indicated the positive effect of the improvement in reproductive management of dairy herd on the net profit and increase in the performance of dairy herds. On the other hand, the net profit of using AI strategies with sexed semen was greater when the size of dairy herds were under expansion (on average from 4 to 67 $/year), but there were no significant differences between the trend in net profit for AI strategies using sexed semen with conception rates of 10 and 20 percentage points lower than the rate in conventional semen. In addition, the net

Table 3
The genetic means of milk yield (kg) for breeding cows and heifers with sexed semen (with conception rate of 10% lower than the rate in conventional semen) in different herd sizes (with or without estrus synchronization) over a 15-year period.

Year	Strategy			
	Fixed size	Fixed size and synchronized estrus	Expanding size	Expanding size and synchronized estrus
1	1.59	5.96	0.44	4.60
2	20.10	23.41	19.17	22.82
3	57.14	59.86	56.34	58.38
4	110.51	113.19	109.36	110.12
5	177.32	179.70	177.22	178.00
6	258.95	259.94	259.22	259.92
7	352.96	353.54	354.02	353.65
8	458.52	458.45	460.36	459.50
9	570.82	571.77	576.38	574.32
10	688.59	691.67	698.50	695.05
11	814.68	818.09	827.68	822.65
12	946.09	950.47	963.03	956.82
13	1081.84	1084.24	1102.65	1096.78
14	1223.41	1225.53	1245.54	1240.47
15	1367.85	1370.27	1388.72	1384.86

Table 4
The genetic means of milk yield (kg) for breeding cows and heifers with sexed semen (with conception rate of 20% lower than the rate in conventional semen) in different herd sizes (with or without estrus synchronization) over a 15-year period.

Year	Strategy			
	Fixed size	Fixed size and synchronized estrus	Expanding size	Expanding size and synchronized estrus
1	0.63	7.27	0.47	4.01
2	19.07	25.61	19.53	22.49
3	56.85	61.58	56.63	57.91
4	109.67	115.23	109.63	109.98
5	176.97	181.04	177.16	177.70
6	258.24	261.36	259.00	259.79
7	352.52	355.77	354.47	353.50
8	458.25	460.02	460.57	459.21
9	570.25	573.34	576.42	574.25
10	687.98	693.35	698.97	694.96
11	814.11	820.11	827.50	822.34
12	945.22	951.91	963.75	956.04
13	1081.32	1085.52	1103.07	1096.13
14	1222.19	1226.86	1246.08	1240.30
15	1366.96	1372.17	1389.04	1383.82

Table 5
Net profit of milk production per cow ($) for breeding cows and heifers with conventional semen in different herd sizes (with or without estrus synchronization) over a 15-year period.

Year	Strategy			
	Fixed size	Fixed size and synchronized estrus	Expanding size	Expanding size and synchronized estrus
1	60.28	76.42	3.07	31.40
2	16.22	48.53	5.96	11.77
3	45.54	7.71	-8.53	4.56
4	-12.69	21.72	-3.62	12.10
5	51.98	70.65	6.49	7.82
6	26.78	19.00	-2.94	-1.10
7	27.29	24.86	-4.67	23.17
8	32.37	2.26	5.46	50.25
9	15.05	3.98	35.35	67.00
10	9.54	7.75	13.57	36.37
11	28.14	17.17	17.72	24.57
12	3.16	11.73	42.65	28.81
13	23.68	47.00	39.61	34.42
14	4.91	24.54	42.19	21.95
15	36.18	35.75	27.23	59.06

Table 6
Net profit of milk production per cow ($) for breeding cows and heifers with sexed semen (with conception rate of 10% lower than the rate in conventional semen) in different herd sizes (with or without estrus synchronization) over a 15-year period.

Year	Strategy			
	Fixed size	Fixed size and synchronized estrus	Expanding size	Expanding size and synchronized estrus
1	−51.71	−67.31	203.51	140.68
2	−71.86	−71.69	175.58	301.11
3	−61.83	−41.72	253.92	168.24
4	−14.45	30.63	71.29	57.51
5	88.03	−24.49	43.78	53.99
6	93.59	104.30	62.87	40.67
7	89.63	137.07	173.12	50.83
8	255.29	276.49	122.39	76.57
9	161.16	181.54	109.28	123.37
10	153.98	125.34	60.31	48.14
11	176.86	43.67	127.72	61.91
12	25.77	82.93	105.72	20.78
13	−67.68	−68.18	179.12	196.01
14	−8.42	187.45	29.54	154.92
15	64.00	133.11	127.07	130.43

Table 7
Net profit of milk production per cow ($) for breeding cows and heifers with sexed semen (with conception rate of 20% lower than the rate in conventional semen) in different herd sizes (with or without estrus synchronization) over a 15-year period.

Year	Strategy			
	Fixed size	Fixed size and synchronized estrus	Expanding size	Expanding size and synchronized estrus
1	−62.68	−63.12	74.46	48.48
2	−72.79	−68.22	206.07	192.02
3	37.69	−74.44	314.28	260.81
4	68.94	−62.61	65.89	96.56
5	−9.44	−36.75	28.55	99.32
6	1.54	106.47	15.73	60.70
7	209.79	60.45	90.84	90.22
8	210.55	299.69	38.53	−38.81
9	119.06	171.66	100.22	73.19
10	207.73	149.61	230.04	183.59
11	69.67	24.21	106.92	220.43
12	50.19	92.07	207.12	136.81
13	14.20	129.34	53.64	37.94
14	21.34	99.47	69.07	50.46
15	101.52	77.51	95.86	120.56

profit for using sexed semen with conception rates 10 percentage points lower than the rate of conventional semen was generally greater than the net profit of using sexed semen with conception rates of 20 percentage points lower than the rate of conventional semen at the end of the time horizon. The possible reason for this result was due to the variation in the conception rates (20 or 30%) for the sexed semen used in the current study. In other words, the use of sexed semen with conception rate of 30% is likely to result in lower insemination costs and fertility problems than its counterpart with conception rate of 20%. The results of sensitivity analysis of the model have not been shown in the manuscript, but these indicated that milk sales income and feed costs were the most important revenue and cost variables, respectively, so that a 20% increase or decrease in milk sales income and feed costs have a significant effect on the net profit for AI strategies using either sexed or conventional semen. Also, 20% increase in the price of heifers resulted in a relatively consistent increase in the net profit. This could be important for AI strategies with sexed semen. The net profit of dairy herds did not change significantly when other input variables varied by 20%. Other economic benefits of sexed semen may result from higher prices for female dairy calves compared with male dairy calves, the added value of (crossbred) calves not used as replacements, optimized herd turnover rates, reduced dystocia, increased rate of genetic progress, and possibly improved biosecurity if previously “open” herds could be “closed” (De Vries et al., 2008).

Because of the reduced fertility of sexed semen compared with conventional semen, the heifers are the primary candidates for sexed semen (Weigel, 2004) because they have greater fertility than cows (Garner and Seidel, 2003), female calves reduce the risk of dystocia (Seidel, 2003), and heifers are on average genetically superior to cows (Hohenboken, 1999). Predictions in the scientific literature regarding the impact of sexed semen on genetic progress vary widely, as do projections of its impact on the profitability of dairy herds. The results of this study were consistent with the results of other studies reported the rate of genetic change can be expected to increase by not more than 15% as a result of increased selection of dams through sexed semen (Hohenboken, 1999; Weigel, 2004).

One special case of non-equilibrium application in the current study is herd expansion. Increasing herd size by having more female calves is very attractive in many situations, and may become even more attractive due to biosecurity issues in large herds. There is considerable published information about the value of sexed sperm in equilibrium simulations (Hohenboken, 1999; Taylor et al., 1988). The results of current study, showing a higher economic value of sexed semen than that of conventional semen were consistent with the reports of other studies (Ettema et al., 2007; Amann, 1999; Taylor et al., 1988; Van Vleck and Everett, 1976). The break-even prices of female calves and insemination costs for AI strategies were not determined in the current study, but Olynk and Wolf (2007) established break-even heifer calf prices and break-even insemination costs for the use of sexed semen in dairy heifers compared to conventional semen. When conception risk to first service for conventional semen was 58% and decreasing after first service, the profit per heifer enrolled was $208. When conception rates for sexed semen were 75% of those of conventional semen and the first service was with sexed semen, the additional profit was $5. Second and later services with sexed semen were not profitable. Under these conditions, break-even heifer calf values were nearly $500. Break-even sexed semen insemination costs were close to $50. Also, consistent with the current results, Seidel (2003) hypothesized that with near normal fertility a premium in the range of $10 per dose could be carried by sexed semen.

4. Conclusions

The results of this study indicated that a rate of genetic progress in the AI program with sexed semen was significantly greater than the rate of progress in AI population with conventional semen. The trend of net profit of using AI strategies with conventional semen over the 15-year horizon was greater when herds were expanding. In addition, the sex-controlled scenarios resulted in many more female calves than sex-uncontrolled scenarios. This allows the farmer to select animals with the best genetic potential as dairy replacement heifers, thus increasing the rate of genetic gain in the dairy herd. Also, the trend of net profit over the time horizon for AI strategies with sexed semen was greater when the size of herd was fixed and the estrous cycles of the cows and heifers were synchronized. The net profit of using AI with sexed semen was greater than that of AI with conventional semen at the end of the time horizon.

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Measurement of interferon-tau (IFN-τ) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18–20 d after insemination in dairy cattle

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ABSTRACT

The objective was to diagnose pregnancy within 18 d after insemination by measuring interferon-tau (IFN-τ)-stimulated genes (ISG) expression in circulatory leukocytes. Based on microarray results, three genes were selected [2′-5′ oligoadenylate synthetase 1(Oas1), myxovirus resistance gene 2 (Mx2), and interferon-stimulated gene 15 kDa protein (Isg15)] because they were known to be interferon-stimulated genes (ISG) and were also differentially expressed in the samples. The respective genes were assayed by using real time reverse transcriptase polymerase chain reaction (RTPCR). In the first experiment, RNA was isolated from leukocytes of pregnant ($n = 5$) and non-pregnant ($n = 15$) dairy cows on each of d 14, 16, 18, and 20 after insemination. The ISG expression (Mx2 and Isg15) was greater in pregnant compared with non-pregnant cows on d 18 and 20. In the second experiment, we attempted to define the shortest interval to pregnancy detection by measuring ISG on d 17 (Exp. 2A) or d 18 (Exp. 2B). For Exp. 2A, blood was collected from pregnant ($n = 16$) and non-pregnant ($n = 16$) cows on d 17 after insemination. On d 17, pregnant cows had greater Mx2 and Oas1 but Receiver Operator Characteristic (ROC) curves showed that the increase was too small to develop a suitable cut-off for a reliable pregnancy test. For Exp. 2B, blood samples were collected from pregnant ($n = 21$) and non-pregnant ($n = 21$) cows on d 18 after insemination. For both Mx2 and Oas1, primiparous pregnant cows had greater ISG expression than primiparous non-pregnant cows on d 18. For multiparous cows, however, pregnant and non-pregnant cows were similar for ISG expression on d 18. The possibility of correcting for pre-insemination ISG expression was examined in a final series of experiments. Blood samples were collected from cows ($n = 54$) and heifers ($n = 24$) during the luteal phase preceding insemination. A second sample was collected from the same cows [pregnant ($n = 13$) and non-pregnant ($n = 41$)] and heifers [pregnant ($n = 17$) and non-pregnant ($n = 7$)] 18 d after insemination. The ratio of the second sample (after insemination) to the first sample (before insemination) minimally increased the sensitivity of the ISG test for pregnancy detection. There was a large increase in ISG expression on d 18 of pregnancy in heifers (approximately 1 year of age) that could be measured for a reliable pregnancy test. The conclusion from the studies is that a reliable ISG-based method for pregnancy detection could be applied as early as d 18 but only in heifers. Older cows have a lesser response that reduces the sensitivity of the ISG test on or before d 18.

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1. Introduction

The implementation of controlled reproductive management programs that include estrous synchronization or timed artificial insemination can decrease days open by controlling the interval from calving to first insemination (Tenhagen et al., 2004). A method of re-synchronization of estrus is typically used in these herds so that the second insemination can be uniformly applied to cows that are not pregnant after the first insemination. The re-synchronization program is generally implemented so that the second insemination is performed at 28–56 d after first insemination (i.e., a 4–8-wk interval). Regardless of the interval, cows must undergo pregnancy diagnosis because re-synchronization programs typically employ an injection of $PGF_{2\alpha}$ that will cause an abortion if the cow is pregnant. Non-pregnant cows could be re-inseminated at a shorter interval (for example 3 wk after first insemination) if a pregnancy diagnosis could be performed sooner after insemination. Such a 3-wk system was proposed by Lucy et al. (2004). The system requires a method of pregnancy detection that is accurate on or before 18 d after first insemination; a time when ultrasonography cannot reliably detect pregnancy. A chemical method of pregnancy detection, therefore, is necessary.

One candidate for chemical pregnancy detection within 18 d after insemination is interferon-tau (IFN-τ), a well-characterized protein produced by the ruminant trophoblast beginning on d 14 of pregnancy (Thatcher et al., 1995). Some IFN-τ escapes the uterus and it can be detected in blood (Oliveira et al., 2008; Bott et al., 2010). Circulating concentrations of IFN-τ are extremely low, however, and this fact precludes their use in a blood pregnancy test. An alternative is to measure the response of circulatory leukocytes to IFN-τ. Leukocytes respond to IFN-τ by expressing interferon-stimulated genes (ISG). The utility of ISG for the purpose of pregnancy detection in dairy cattle has been demonstrated recently (Han et al., 2006; Gifford et al., 2007; Stevenson et al., 2007). Han et al. (2006) found that blood progesterone and Isg15 had acceptable results as a method of pregnancy diagnosis if samples were collected over a series of days. Gifford et al. (2007) demonstrated that ISG were up-regulated in peripheral blood leukocytes from pregnant cows on d 18 and 20 after AI compared with d 0 (day of AI). Stevenson et al. (2007), however, concluded that Mx2 was not a viable marker for pregnancy diagnosis in dairy heifers.

The objective of this study was to determine the utility of ISG to diagnose pregnancy within 18 d after insemination. We identified candidate ISG in a microarray experiment and then performed a series of *in vivo* experiments to determine the utility of ISG for early pregnancy detection in lactating dairy cows and non-lactating dairy heifers.

2. Materials and methods

2.1. Microarray analysis

Three primiparous lactating Holstein cows from the University of Missouri Foremost Dairy Farm (a typical confinement-style dairy; milked twice daily, housed in a freestall barn, fed a totally mixed ration, etc.) were estrus-synchronized and inseminated by using the Presynch–Ovsynch program (Moreira et al., 2001). Blood was collected from the median caudal vein or artery into tubes containing 100 μl of a 15% solution of EDTA (K_3) (Tyco Healthcare Group, Mansfield, MA) on d 15 and on d 18 after AI. The blood was placed on ice and the RNA isolation from leukocytes began within 2 h of blood collection. The RNA was extracted and purified using the QIAamp® RNA blood kit (Qiagen, Austin, TX) and subjected to DNase treatment according to the manufacturer's instructions. Purified RNA was dissolved in sterile water and its concentration was determined by measuring absorbance at 260 nm. The purity of RNA was determined by calculating the ratio of absorbencies at 260 and 280 nm using a ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). 1 μg of RNA was electrophoresed through a 1% agarose gel in Tris–borate/EDTA buffer (0.09 M Tris–borate and 0.002 M EDTA) with ethidium bromide (0.5 μg/ml) to verify the integrity of each sample. Isolated RNA was stored at −80 °C. The cows were then examined by ultrasonography to confirm that they were pregnant at the time of blood collection.

The RNA was submitted to the DNA core facility at the University of Missouri for microarray analysis. 0.5 μg of total RNA was used to make the biotin-labeled antisense RNA (aRNA) target using the MessageAmp™ Premier RNA amplification kit (Ambion, Austin, TX) by following the manufacturer's procedures. Briefly, the total RNA was reverse transcribed to first strand cDNA with a oligo(dT) primer bearing a 5′-T7 promoter using ArrayScript reverse transcriptase (Ambion). The first strand cDNA then underwent second-strand synthesis to convert into double stranded cDNA template for *in vitro* transcription. The biotin-labeled aRNA was synthesized using T7 RNA transcriptase with biotin–NTP mix. After purification, the aRNA was fragmented in fragmentation buffer at 94 °C for 35 min. 130 μl of hybridization solution containing 50 ng/μl of fragmented aRNA was hybridized to the Bovine Genome Array Genechip (Affymetrix, Santa Clara, CA) at 45 °C for 20 h. After hybridization, the chips were washed and stained with R-phycoerythrin–streptavidin on Affymetrix fluidics station 450 using fluidics protocol Midi_euk2v3-450. The image data were acquired by Affymetrix Genechip scanner 3000. Data were analyzed by using ArrayStar 2.0 software (DNASTAR Inc., Madison, WI).

2.2. Experiment 1 (ISG expression from d 14 to 20 after insemination)

Lactating Holstein cows ($n = 20$) at the University of Missouri Foremost Dairy Farm were used. Cows were synchronized for timed AI by using Ovsynch (Pursley et al., 1997). Blood samples were collected 14, 16, 18, and 20 d after AI by using the methods described for the microarray experiment (preceding section). The RNA was extracted from leukocytes and DNase-treated according to the procedures used for the microarray. A portion of the blood sample was used for plasma isolation for progesterone analysis by radioimmunoassay (Kirby et al., 1997; single

Table 1
Gene, GenBank number, primer sequence (forward and reverse primers; 5′ to 3′) and the location of the primer within the GenBank sequence for genes amplified during the RTPCR.

Gene	GenBank	Primer	Primer sequence	Primer location
Isg15	NM_174366	Forward	5′-CAGCCAACCAGTGTCTGCAGAGA-3′	14–36
		Reverse	5′-CCAGGATGGAGATGCAGTTCTGC-3′	284–306
Mx2	NM_173941	Forward	5′-CTTCAGAGACGCCTCAGTCG-3′	2071–2090
		Reverse	5′-TGAAGCAGCCAGGAATAGTG-3′	2283–2302
Oas1	NM_001040606	Forward	5′-ACCCTCTCCAGGAATCCAGT-3′	1157–1176
		Reverse	5′-GATTCTGGTCCCAGGTCTGA-3′	1336–1355
Cyclophilin A	NM_178320	Forward	5′-CACCGTGTTCTTCGACATCG-3′	23–42
		Reverse	5′-ACAGCTCAAAAGAGACGCGG-3′	65–84

assay with an intra-assay CV of 7.6%). Cows were diagnosed for pregnancy after the blood collection phase when they were 30 d after insemination. Five cows were diagnosed pregnant and 15 were not pregnant at 30 d.

Total RNA from leukocytes was transcribed into cDNA by using the SuperScript^{TM} First Strand RT-PCR kit (Invitrogen, Carlsbad, CA). The RTPCR reactions were conducted using a cDNA equivalent of 25 ng of total RNA. The cDNA was amplified in an ABI Prism 7500 thermocycler (Applied Biosystems, Foster City, CA) using the QuantiTect Sybr Green PCR kit (Qiagen) and specified primers for Isg15 and Mx2 (Table 1). Cyclophilin A was also analyzed and used as an internal control housekeeping gene. All of the PCR products were DNA sequenced and had >98% homology with their target gene. Each PCR 96-well plate contained a "no template control" (water substituted for cDNA in the reaction) to insure that there was no background contamination. Samples were assayed in triplicate.

Gene expression data were expressed as fold change relative to a standard control sample that was run within each assay plate. The ISG fold change was also expressed as a ratio to cyclophilin fold change (Isg15_ratio and Mx2_ratio). Gene expression and plasma progesterone concentrations were analyzed by using Proc Mixed of SAS (SAS Institute Inc., Cary, NC). The statistical model included: pregnancy status, day after insemination and the pregnancy status by day interaction. Day was defined as the repeated variable, cow nested within pregnancy status was defined as the subject variable (random), and the covariance structure was heterogeneous compound symmetry. The lsmeans were separated by using the pdiff procedure within Proc Mixed.

2.3. Experiment 2 [ISG expression on d 17 (Exp. 2A) or 18 (Exp. 2B) after AI]

Lactating Holstein cows from the University of Missouri Dairy Farm were used. Blood samples were collected on d 17 (Exp. 2A; $n = 32$) or 18 (Exp. 2B; $n = 42$) after AI and processed for RNA and plasma isolation by using the methods described in the previous sections. The plasma progesterone analyses had an intra-assay CV of 10.0% (Exp. 2A) and 14.5% (Exp. 2B). Cows were diagnosed as pregnant [$n = 16$ (Exp. 2A); $n = 21$ (Exp. 2B)] or not pregnant [$n = 16$ (Exp. 2A); $n = 21$ (Exp. 2B)] by transrectal ultrasonography on d 32 after insemination.

Total leukocyte RNA was transcribed into cDNA by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The RTPCR reactions were carried out using a cDNA equivalent of 25 ng of total RNA. The RTPCR were done in an ABI Prism 7500 thermocycler (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primers for Oas1, Mx2, and cyclophilin (Table 1). Background contamination was monitored by using a no template control. Samples were assayed in duplicate.

Gene expression data were expressed as fold change relative to a standard control sample that was run within each assay plate. The ISG fold change was also expressed as a ratio to cyclophilin fold change (Oas1_ratio and Mx2_ratio). Statistical analyses were done by using Proc GLM of SAS. The statistical model included the effects of pregnancy status, parity [primiparous (i.e., first parity) or multiparous (second or greater parity)] and the status by parity interaction. Receiver operator characteristic (ROC) curves were generated in SAS as described by Mandrekar and Mandrekar (2005).

2.4. Experiment 3 (ISG expression in the luteal phase of the preceding cycle and d 18 after AI)

Lactating Holstein cows ($n = 54$; Exp. 3A) were selected from a private dairy in northeast Missouri. Holstein heifers ($n = 24$; Exp. 3B) were from the University of Missouri Foremost Dairy Farm. The private dairy was a typical large confinement herd (approximately 2500 lactating cows with totally mixed ration feeding, thrice daily milking, freestall housing, and compost bedding). Heifers from the Foremost Dairy Farm were housed in a pen with sand-bedded freestalls and fed hay as well as orts from the lactating cow pens.

For lactating cows, the first sample collection was done at approximately 80 d postpartum immediately preceding the final injection of PGF$_{2\alpha}$ of a Presynch–Ovsynch timed AI program (Moreira et al., 2001). Based on a theoretical response to the program, the cows were at approximately d 18 of the estrous cycle at the time of sample collection. The cows were screened for plasma progesterone concentration and had to have $\geq$1.0 ng/ml of plasma progesterone at first sample collection in order to qualify for the second sample collection. The second sample collection was performed at d 18 after insemination. Blood samples were analyzed as described for Exp. 2, except that the first phase

Table 2

Affymetrix probe ID, GenBank number, fold change in gene expression [up (increased) or down (decreased)], *P*-value, and gene name for genes that were highly expressed (>100 expression level) in RNA isolated from leukocytes and for which gene expression was at least twofold up or down-regulated in blood samples collected on d 15 (reference day) and d 18 of pregnancy.

Affymetrix probe	GenBank	Fold change	*P*	Gene name (symbol)
Bt.20891.1.S1_at	NM_001040606	7.5 up	<0.019	2′,5′-Oligoadenylate synthetase 1, 40/46 kDa (OAS1)
Bt.15788.3.S1_at	BC149213	5.3 up	<0.029	Similar to bone marrow stromal cell antigen 2 (LOC507402)
Bt.12304.1.S1_at	NM_174366	5.2 up	<0.037	ISG15 ubiquitin-like modifier (ISG15)
Bt.17729.1.A1_at	XM_872122	4.4 up	<0.033	Interferon-induced protein 44 (IFI44)
Bt.29258.1.A1_a_at	NM_001037821	3.6 up	<0.042	Mucosal vascular addressin cell adhesion molecule 1 (MADCAM1)
Bt.8143.1.S1_at	NM_173941	3.5 up	<0.009	Myxovirus resistance 2 (mouse) (MX2)
Bt.8436.1.S1_at	NM_001075588	3.0 up	<0.020	Interferon, alpha-inducible protein 6 (IFI6)
Bt.23597.1.S1_at	NM_001114515	2.5 up	<0.036	Nuclear protein, transcriptional regulator, 1 (NUPR1)
Bt.4675.1.S1_a_at	NM_173940	2.0 up	<0.010	Myxovirus resistance 1, interferon-inducible protein p78 (mouse) (MX1)
Bt.19295.1.S1_at	NM_001101180	2.0 up	<0.032	Apolipoprotein L domain containing 1 (APOLD1)
Bt.5002.1.S1_at	NM_001035354	2.1 down	<0.041	Glycerol-3-phosphate dehydrogenase 1 (soluble) (GPD1)
Bt.26168.1.A1_at	CK775191	2.1 down	<0.040	Unknown
Bt.29753.1.S1_x_at	XM_001251207	2.2 down	<0.024	Similar to T-cell receptor beta chain V region CTL-F3
Bt.24554.1.S1_at	XM_583848	2.2 down	<0.050	Similar to zinc finger protein 212 (ZNF212)
Bt.16727.2.S1_at	NM_001075941	2.3 down	<0.005	Ceroid-lipofuscinosis, neuronal 8 (CLN8)
Bt.7072.1.S1_at	XM_001256316	2.3 down	<0.007	Similar to zinc finger protein 524 (ZNF524)
Bt.26530.3.S1_a_at	XM_001788744	2.6 down	<0.020	Similar to acyl-CoA dehydrogenase family, member 10 (ACAD10)

Table 3

Relative gene expression (lsmean ± SEM) for interferon-stimulated genes and blood progesterone concentration (ng/ml) on d 17 or 18 after AI for cows (Exp. 2A, 2B, and 3A) or nulliparous heifers (Exp. 3B).

	Primiparous		Multiparous		*P*		
	Not pregnant	Pregnant	Not Pregnant	Pregnant	Status	Parity	S × P
Day 17 (Exp. 2A)							
N	8	8	8	8			
Mx2[a]	2.5 ± 0.7	4.9 ± 1.1	2.1 ± 0.7	2.9 ± 0.7	<0.056	NS[b]	NS
Oas1	1.6 ± 0.7	3.9 ± 1.0	0.9 ± 0.7	2.4 ± 0.6	<0.018	NS	NS
Cyclophilin	4.8 ± 1.0	5.6 ± 1.5	5.6 ± 1.0	5.8 ± 0.8	NS	NS	NS
Mx2_ratio[a]	0.6 ± 0.2	1.3 ± 0.3	0.5 ± 0.2	0.6 ± 0.2	<0.105	NS	NS
Oas1_ratio	0.4 ± 0.2	0.9 ± 0.3	0.2 ± 0.2	0.5 ± 0.1	<0.045	NS	NS
Progesterone	3.6 ± 0.7	4.6 ± 1.1	3.2 ± 0.8	4.6 ± 0.6	NS	NS	NS
Day 18 (Exp. 2B)							
N	10	6	11	15			
Mx2	9.4 ± 4.0	32.2 ± 5.2	5.3 ± 3.8	8.2 ± 3.3	<0.004	<0.002	<0.021
Oas1	2.4 ± 1.3	7.5 ± 1.5	1.0 ± 1.1	2.0 ± 0.9	<0.012	<0.005	<0.083
Cyclophilin	10.3 ± 2.0	13.1 ± 2.5	9.4 ± 1.9	14.0 ± 1.6	<0.074	NS	NS
Mx2_ratio	0.7 ± 0.2	2.3 ± 0.3	0.6 ± 0.4	0.7 ± 0.2	<0.001	<0.001	<0.005
Oas1_ratio	5.9 ± 2.4	19.9 ± 3.1	3.1 ± 2.3	5.1 ± 1.9	<0.003	<0.001	<0.020
Progesterone	3.5 ± 0.5	5.0 ± 0.6	2.5 ± 0.5	3.2 ± 0.4	<0.036	<0.009	NS
Day 18 (Exp. 3A)							
N	13	11	28	2			
Mx2	0.6 ± 0.1	1.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.3	NS	<0.011	<0.017
Oas1	0.7 ± 0.2	1.6 ± 0.2	0.7 ± 0.1	0.3 ± 0.5	NS	<0.017	<0.021
Cyclophilin	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.2	NS	NS	NS
Mx2_ratio	0.4 ± 0.1	1.0 ± 0.1	0.5 ± 0.1	0.2 ± 0.3	NS	<0.026	<0.015
Oas1_ratio	0.5 ± 0.2	1.3 ± 0.2	0.6 ± 0.1	0.2 ± 0.5	NS	<0.074	<0.037
Progesterone	4.6 ± 0.8	6.5 ± 0.9	3.5 ± 0.5	5.6 ± 2.0	NS	NS	NS

Day 18 (Exp. 3B)	Nulliparous		*P*
	Not pregnant	Pregnant	Status
N	7	17	
Mx2	1.0 ± 0.9	4.4 ± 0.6	<0.004
Oas 1	1.9 ± 1.6	9.3 ± 1.1	<0.001
Cyclophilin	1.0 ± 0.1	1.1 ± 0.1	NS
Mx2_ratio	0.9 ± 0.8	4.2 ± 0.5	<0.003
Oas1_ratio	1.7 ± 1.5	8.9 ± 1.0	<0.001
Progesterone	4.7 ± 1.1	10.4 ± 0.7	<0.001

[a] Exp. 2A: Mx2 and Mx2_ratio had fewer observations ($n = 3$) for primiparous pregnant cows.

[b] Not significant ($P > 0.10$).

of the RNA isolation process (leukocyte isolation and lysis) was performed at the private farm. The cell lysate samples were kept on dry ice until they arrived back at the laboratory when they were moved to −80 °C storage until further processing. Another sample of whole blood was left on ice and was immediately processed for plasma and stored as described above. Cows were diagnosed as pregnant by transrectal ultrasonography on d 39 after insemination. There were 13 pregnant and 41 non-pregnant cows.

The heifers used for Exp. 3B were synchronized for first insemination by using a CIDR device (Pfizer Animal Health, New York, NY) inserted for 14 d and an injection of $PGF_{2\alpha}$ (25 mg Lutalyse, Pfizer Animal Health) 16 d after CIDR device removal. The AI was done 12 h after an observed estrus. The first sample collection was immediately before the injection of $PGF_{2\alpha}$ when the heifers were theoretically in the late luteal phase (d 13–15 of the estrous cycle). The heifers were screened for plasma progesterone concentration and had to have ≥1.0 ng/ml of plasma progesterone at first sample collection in order to qualify for the second sample collection. The second sample was collected 18 d after AI. Samples were processed and analyzed as described in Exp. 3A. Heifers were diagnosed for pregnancy between d 31 and 34 after AI and there were 17 pregnant and 7 non-pregnant heifers.

Gene expression data were analyzed by using two different methods. For the first set of analyses, only the data from the second sample (d 18 after insemination) were analyzed. For the second set of analyses, a ratio of sample 2 (taken 18 d after AI) to sample 1 (taken before first AI) was analyzed. The variable names "Mx2/Mx2", "Oas1/Oas1", and "Cyclo/Cyclo" were used to define the sample 2 to sample 1 ratio. Regardless of the approach, the data were analyzed using Proc GLM in SAS. The model included status, parity, and status by parity (Exp. 3A) or status alone (Exp. 3B). The ROC curves were generated as described in Exp. 2.

3. Results

3.1. Microarray experiment

17 genes had a greater than or equal to 2-fold change in gene expression (increase or decrease; Table 2) on d 18 compared with d 15 of pregnancy. Among the genes with ≥2-fold increase were genes for which expression was known to be stimulated by IFN-τ [Oas1 (7.5-fold increase), Isg15 (5.2-fold increase), Mx2 (3.5-fold increase), and Mx1 (2.0-fold increase)].

3.2. Experiment 1

There was a status by day interaction ($P < 0.001$) for Mx2_ratio (Fig. 1A). The Mx_2 ratio increased from 14 to 20 d in pregnant cows but remained unchanged in non-pregnant cows in the same period. The pattern of gene expression for Isg15_ratio (Fig. 1B) was similar to Mx2_ratio (status by day interaction; $P < 0.007$). The regression of Mx2_ratio on Isg15_ratio was significant ($R^2 = 0.87$; $P < 0.001$; Mx2_ratio = 0.36 + 0.51 × Isg15_ratio). Plasma progesterone concentrations were not different for pregnant and non-pregnant cows ($P > 0.10$; Fig. 1C).

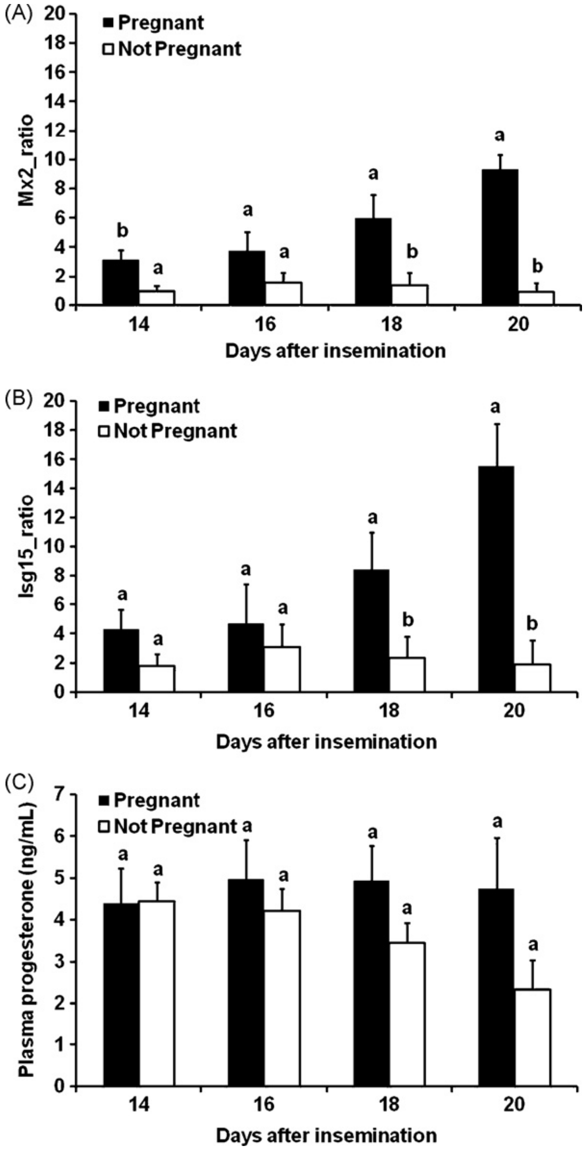

Fig. 1. Ratio of Mx2 fold change to cyclophilin fold change (Mx2_ratio; A), Isg15 fold change to cyclophilin fold change (Isg15_ratio; B), and plasma progesterone concentrations (C) on d 14–20 after insemination in dairy cows that were later diagnosed as pregnant or not pregnant to insemination. Bars within day with different superscript differ ($P < 0.05$).

3.3. Experiment 2A (blood sample collection on d 17)

Gene expression of Mx2 tended to be greater for pregnant compared with non-pregnant cows when expressed as unadjusted ($P < 0.056$) or as a ratio to cyclophilin (Mx2_ratio; $P < 0.105$; Table 3). Likewise, Oas1 expression was greater in pregnant compared with non-pregnant cows (Oas1, $P < 0.018$; Oas1_ratio, $P < 0.045$). Neither cyclophilin nor progesterone was affected by pregnancy status ($P > 0.10$). There was no effect of parity or status by parity interaction for Mx2, Oas1, Mx2_ratio, Oas1_ratio, cyclophilin or progesterone. The ROC curves for Oas1_ratio and Mx2_ratio on d 17 (Fig. 2A and B) were comprised of a diagonal across the middle of the

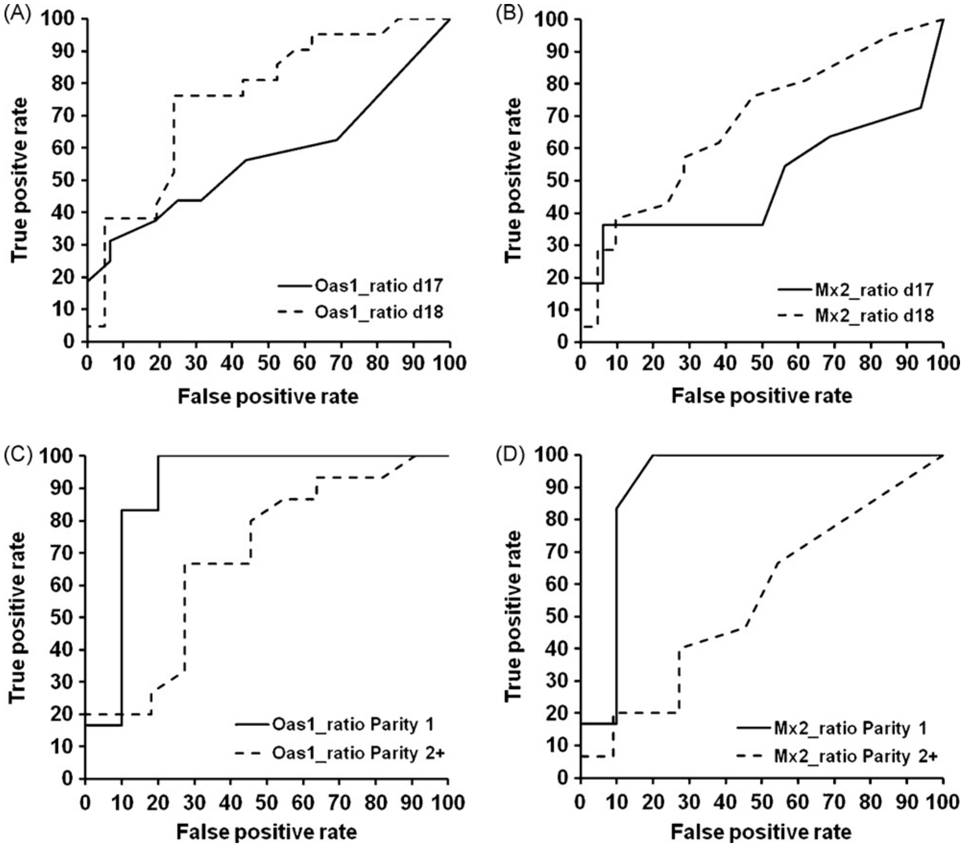

Fig. 2. Receiver operator characteristic (ROC) curves for Oas1 and Mx2 on d 17 and 18 (A, Oas1_ratio. B, Mx2_ratio; Exp. 2) and for different parities on d 18 after insemination (C, Oas1_ratio. D, Mx2_ratio; Exp. 2). The ROC curve is a graphical depiction of the true positive rate and the false positive rate when an increasing cut-off for the test is applied. If both the true positive rate and the false positive rate decrease when the cut-off is increased then the graph has a diagonal line through its center and the test is not useful (no better than flipping a coin). Deflection of the line to the left of center is an indication of a useful test because it has a relatively high true positive rates and low false positive rates at a given cut-off. The ideal test has an ROC curve that is fully deflected to the left with a cut-off that achieves a 100% true positive and 0% true negative rate.

plot demonstrating that a suitable cut-off (one that possessed a 100% true positive rate and also a 0% false positive rate) could not be established. The regression of Mx2_ratio on Oas1_ratio was significant ($R^2 = 0.82$; $P < 0.001$; Mx2_ratio = 0.20 + 1.15 × Oas1_ratio).

3.4. Experiment 2B (blood sample collection on d 18)

There was a status by parity interaction for both Mx2 (Mx2, $P < 0.021$; Mx2_ratio, $P < 0.005$) and Oas1 (Oas1, $P < 0.083$; Oas1 ratio, $P < 0.020$; Table 3). Primiparous pregnant cows had greater ISG expression than primiparous non-pregnant cows. Expression of these genes in multiparous pregnant cows was similar to multiparous non-pregnant cows. Plasma progesterone concentrations were greater in primiparous cows than multiparous cows (effect of parity; $P < 0.009$) and greater in pregnant than non-pregnant cows (effect of status, $P < 0.036$). The ROC curves for Oas1_ratio and Mx2_ratio on d 18 (Fig. 2A and B) were improved relative to Exp. 2A (i.e., the curves were deflected to the left relative to d 17) but failed to demonstrate a usable cut-off for the test. Parity affected the shape of the ROC curves (Fig. 2C and D). Primiparous cows had an ROC curve that was superior to multiparous cows. Data derived from primiparous cows achieved a 100% true positive rate with a false positive rate of less than 30% for both Oas1_ratio and Mx2_ratio. The regression of Mx2_ratio on Oas1_ratio was significant ($R^2 = 0.76$; $P < 0.001$; Mx2_ratio = 0.28 + 0.09 × Oas1_ratio).

3.5. Experiment 3A (multiple sample collection in lactating cows)

There was no effect of status, parity, or status by parity for Mx2, Mx2_ratio or Oas1_ratio for the sample collected before insemination (all cows not pregnant). Parity (but not status or status by parity) was significant for Oas1 before insemination ($P < 0.045$; 0.9 ± 0.1 versus 0.4 ± 0.2 for primiparous and multiparous cows, respectively). For the sample collected on d 18 after insemination, there was a status by parity interaction for both Mx2 (Mx2, $P < 0.017$; Mx2_ratio, $P < 0.015$) and Oas1 (Oas1, $P < 0.021$; Oas1_ratio, $P < 0.037$; Table 3). Primiparous pregnant cows had greater ISG expression than primiparous non-pregnant cows. Multiparous pregnant cows were similar to multiparous non-pregnant cows in the expression of these genes. Plasma progesterone concentrations were similar in primiparous and multiparous cows and were not affected by

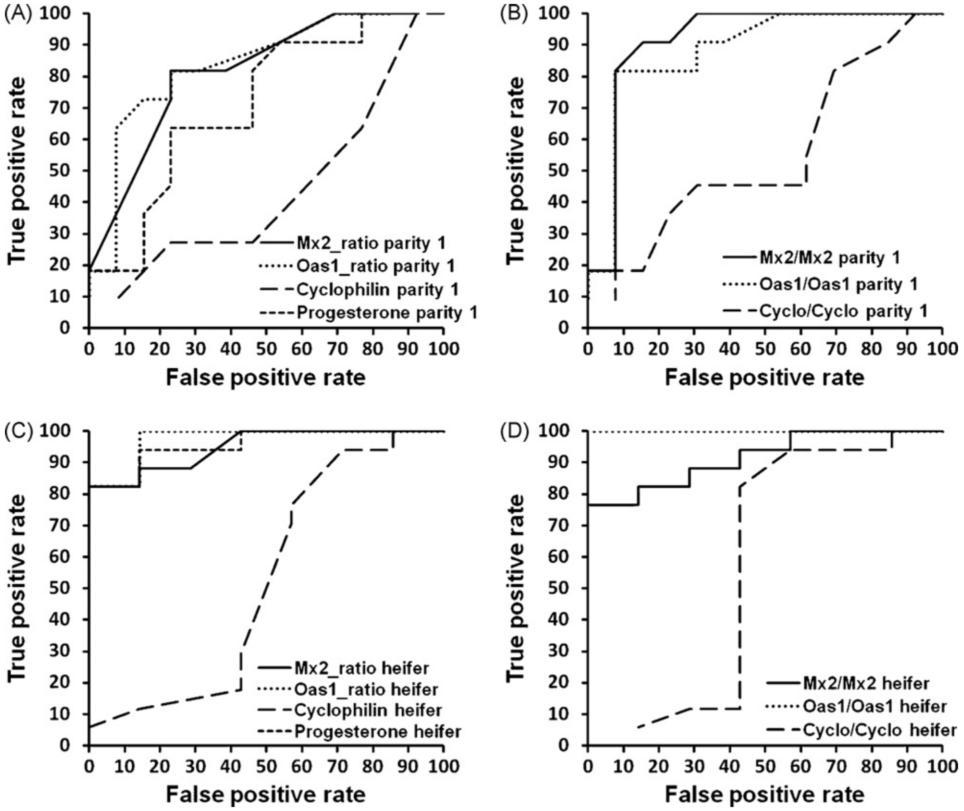

Fig. 3. Receiver operator characteristic (ROC) curves for Oas1, Mx2, cyclophilin, and progesterone on d 18 after AI in primiparous cows (A) and nulliparous heifers (C), and the ROC curves for the ratio of sample 2 to sample 1 for Mx2, Oas1, and cyclophilin in primiparous cows (B) and nulliparous heifers (D). The ROC curve is a graphical depiction of the true positive rate and the false positive rate when an increasing cut-off for the test is applied. If both the true positive rate and the false positive rate decrease when the cut-off is increased then the graph has a diagonal line through its center and the test is not useful (no better than flipping a coin). Deflection of the line to the left of center is an indication of a useful test because it has a relatively high true positive rates and low false positive rates at a given cut-off. The ideal test has an ROC curve that is fully deflected to the left with a cut-off that achieves a 100% true positive and 0% true negative rate.

pregnancy status. Although primiparous pregnant cows had greater ISG expression, the ROC curves for Mx2_ratio and Oas1_ratio in primiparous cows failed to demonstrate a usable cut-off for the test (Fig. 3A). As expected, cyclophilin (control housekeeping gene) demonstrated minimal utility for a pregnancy test (Fig. 3A). Using plasma progesterone concentrations on d 18 for pregnancy diagnosis yielded an ROC curve that was nearly identical to Mx2_ratio and Oas1_ratio.

When the ratios of gene expression [sample 2 (after AI) divided by sample 1 (before AI)] were analyzed (Table 4), there was a status by parity interaction for

Table 4
Ratio of gene expression for MX2, Oas1, and cyclophilin in sample 1 (collected during the luteal phase of the estrous cycle before AI) to sample 2 (collected d 18 after AI) in cows (Exp. 3A) and heifers (Exp. 3B).

Day 18 (Exp. 3A)	Primiparous		Multiparous		*P*		
	Not pregnant	Pregnant	Not pregnant	Pregnant	Status	Parity	S × P
N	13	11	28	2			
Mx2/Mx2	1.0 ± 0.5	2.8 ± 0.5	1.8 ± 0.3	0.8 ± 1.2	NS[a]	NS	<0.071
Oas1/Oas1	0.9 ± 0.8	3.0 ± 0.9	2.3 ± 0.5	0.9 ± 2.0	NS	NS	NS
Cyclo/Cyclo	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.3	NS	NS	NS

Day 18 (Exp. 3B)	Nulliparous		*P*
	Not pregnant	Pregnant	Status
N	7	17	
Mx2/Mx2	1.8 ± 1.4	6.1 ± 0.9	<0.015
Oas1/Oas1	1.0 ± 1.3	6.3 ± 0.8	<0.003
Cyclo/Cyclo	0.9 ± 0.1	0.9 ± 0.1	NS

[a] Not significant ($P > 0.10$).

Mx2/Mx2 ($P \leq 0.071$) but the status by parity interaction for Oas1/Oas1 was not significant ($P = 0.156$). For Mx2/Mx2, primiparous pregnant cows had greater Mx2 expression when compared with primiparous non-pregnant cows. The Mx2/Mx2 for multiparous cows, however, was similar in pregnant and non-pregnant cows. As expected, there was no effect of status, parity, or status by parity on cyclophilin/cyclophilin (control housekeeping gene). The ROC curves for the ratio of sample 2 to sample 1 in primiparous cows (Fig. 3B) were similar in shape relative to those generated in Exp. 2 (Fig. 2C and D) when only a single sample after AI was analyzed. A cut-off was identified for primiparous cows where the true positive rate was 100% while the false positive rate equaled 54% and 31% for Oas1/Oas1 and Mx2/Mx2, respectively.

3.6. Experiment 3B (heifers)

For the sample collected on d 18 after insemination, there was an effect of status for Mx2 ($P < 0.004$), Mx2_ratio ($P < 0.003$), Oas1 ($P < 0.001$), Oas1_ratio ($P < 0.001$), and plasma progesterone ($P < 0.001$) (Table 3). In each analysis, pregnant heifers had greater ISG expression and progesterone than non-pregnant heifers. The ROC curves rising from analyses of a single sample collected 18 d after insemination were similar for progesterone, Mx2_ratio, Oas1_ratio (Fig. 3C). In each case, an 82% true positive rate and 0% false positive rate could be obtained with the appropriate cut-off. When the data were analyzed as ratios of sample 2 (after AI) to sample 1 (before AI) (Table 4), there was an effect of status for Mx2/Mx2 ($P < 0.015$) and Oas1/Oas1 ($P < 0.003$). In each analysis, pregnant heifers had a greater ratio than non-pregnant heifers. Compared with the ROC curves for a single sample collected on d 18 after AI in heifers (Fig. 3C), the ROC curves for a ratio of sample 2 to sample 1(Fig. 3D) were either similar (Mx2) or improved (Oas1). An ideal cut-off for a d 18 pregnancy test was established using Oas1/Oas1 because the true positive rate reached 100% while the false positive rate was 0% (Fig. 3D).

4. Discussion

The rationale for this work was to develop an assay that could be used to detect pregnant cattle before d 18 so that non-pregnant cattle could be synchronized for timed AI on d 21 after first insemination. The system was originally proposed by Lucy et al. (2004). In the proposed system, cattle are diagnosed for pregnancy on d 18 after insemination and non-pregnant cows are injected with $PGF_{2\alpha}$ on the afternoon of d 18 or the morning of d 19 to regress the corpus luteum. 2–2.5 d later (d 21) cattle are injected with GnRH and inseminated. Synchronizing estrus in cows that are late in the estrous cycle takes advantage of the fully mature (in terms of $PGF_{2\alpha}$ response) corpus luteum and the large dominant follicle that is present on the ovary at that time (Lucy, 2007).

The developing conceptus in cattle synthesizes and secretes IFN-τ during maternal recognition of pregnancy. The IFN-τ acts upon circulating blood leukocytes to cause an increase in the expression of a cohort of genes. These IFN-stimulated genes (ISG) can be detected when RNA is isolated from leukocytes and assayed by RTPCR for the purpose of pregnancy diagnosis. In an attempt to discover new ISG, we performed a microarray study of leukocytes collected from pregnant cows on d 15 and 18 after insemination. The microarray study demonstrated that the predominant class of genes that was up-regulated in leukocytes in early pregnant cows is ISG (Table 2). In addition to Isg15 and Mx2 (genes that were known to be increased at this time; Han et al., 2006; Gifford et al., 2007), we identified several other ISG. One of these ISG (Oas1) was known to be stimulated by interferons (Kjaer et al., 2009) but its use in a pregnancy test for cattle had not been previously tested. In this study, we employed two traditional markers (Isg15 and Mx2) and also a new marker (Oas1) for pregnancy in cattle. Cyclophilin was also tested as a control housekeeping gene. In the present study, as expected, cyclophilin was unrelated to the pregnancy status of the animal. The correlation in gene expression for different ISG was typically very high (R^2 greater than 0.75; see for example, Mx2 and Isg15 in Exp. 1 and Mx2 and Oas1 in Exp 2). The high correlation indicates that the ISG are responding to the same signal (IFN-τ) and that assaying multiple ISG for pregnancy detection is not necessary.

In the present study, Isg15 and Mx2 could be detected as early as d 18 after pregnancy (Exp. 1; Fig. 1). At earlier times (d 14 or 16), the ISG were numerically increased but a consistent and significant increase was not detected in ISG in pregnant cows. The difference in ISG gene expression for pregnant and non-pregnant cows appeared to increase with time presumably because of the growing conceptus. The greatest amount of ISG was found on d 20 after insemination in pregnant cows; a time when the conceptus is synthesizing large quantities of IFN-τ (Thatcher et al., 1995). The data for Isg15 and Mx2 were highly correlated ($R^2 = 0.87$). We dropped further analyses of Isg15, therefore, *in lieu* testing for Mx2 (a gene whose expression was known to be increased in pregnant cattle) and Oas1 (a gene for which expression in pregnant cattle had not been evaluated).

In the present study, d 17 was initially targeted for the early pregnancy test. If a test could be developed on this day then inseminated cows could be tested on d 17 and non-pregnant cows could be injected with $PGF_{2\alpha}$ on d 18. Although a significant increase in the mean for ISG on d 17 (Table 3) occurred a viable cut-off for the pregnancy test could not be established. To assess possible cut-offs, ROC curves (Figs. 2 and 3) were generated. The ROC curve is a graphical depiction of the true positive rate and the false positive rate when an increasing cut-off for the test is applied. When the test cut-off is set too low, the true positive rate is 100% (optimal) but the false positive rate is also 100% because all of the non-pregnant cows are called pregnant. Increasing the cut-off (moving to the left on the graph) will decrease the false positive rate. In the optimal scenario, the true positive rate will remain at 100% while the cut-off is increased and the false positive rate will progress toward 0%. If both the true positive rate and the false positive rate decrease when the cut-off is increased then the graph has a diagonal line through its center. This outcome demonstrates that the test is not useful (no better than flipping a coin, per se; see cyclophilin lines in Fig. 3). Lines deflected to the left of center depict tests that have greater utility because they have relatively greater true positive rates and lesser false positive rates at a given cut-off. Applying the aforementioned explanation to the ROC curves from Exp. 2 (d 17, Fig. 2A and B) it was clear that a reliable cut-off could not be established when the test was performed on d 17 after AI.

In the next experiment (Exp. 2B), development of a viable test for d 18 after insemination was attempted. The timing of this test was less desirable because there would be less time to complete the test before the administration of $PGF_{2\alpha}$. We did observe a more robust ISG response on d 18 (Table 3) but, unexpectedly, a parity by status interaction was detected. The interaction appeared to be caused by greater ISG response for primiparous cows when compared with multiparous cows. The increase in ISG was nearly undetectable in the pregnant multiparous cows on d 18.

The reason for the difference in ISG response for the respective parities is unknown. It is possible that the size of the embryo differs from primiparous and multiparous cows and this size difference affects the total capacity of IFN-τ production by the embryo. This hypothesis is supported by the findings of Berg et al. (2010), who found that nulliparous heifers had larger embryos than cows during the period of maternal recognition of pregnancy. Perhaps this difference persists for younger (primiparous) compared with older (multiparous) cows. Alternatively there could be differences in immune function for primiparous and multiparous cows that predispose multiparous cows to a lesser IFN-stimulated response. Another possibility is that the overall larger size of the multiparous cow may create a dilution effect that reduces systemic concentrations of IFN-τ and the ISG response in blood leukocytes.

Regardless of the cause, the fact that multiparous cows failed to have a robust ISG response on d 18 severely limits the utility of the test on d 18. The primiparous cows had an improved response and the ROC curves were superior for primiparous compared with multiparous cows (Fig. 2C and D). Even in the primiparous cows, however, the ROC curves demonstrated that the false positive rate for a pregnancy test would be great. Assuming that a 100% true positive rate is maintained (i.e., all pregnant cows are called pregnant and no pregnant cow is called not pregnant) then 20% of cattle diagnosed pregnant would in reality not be pregnant.

Experiments 2 and 3 employed cows after insemination. One possible explanation for the inability in the present research to establish a reliable test is that some of the cows may have undergone embryonic loss before we categorized them as pregnant or non-pregnant by ultrasonography (after d 30). These cows would theoretically test pregnant on d 17 or 18 but later be diagnosed not pregnant by ultrasonography. There is no simple way to assess how many of these cows with embryonic loss exist and the extent to which they contribute to the false positive rate. A second possibility is that individual cows may have relatively greater or lesser basal ISG gene expression in leukocytes. To examine this possibility, a final experiment was performed in which primiparous and multiparous cows (Exp. 3A) or nulliparous heifers (Exp. 3B) were tested for ISG in the estrous cycle preceding AI and then tested again 18 d after AI. As expected, the amount of ISG expression was similar in pregnant and non-pregnant cattle before insemination (sample 1). The amount of ISG after insemination (sample 2), however, was increased in pregnant cows (Table 3). A status by parity interaction was again detected because the increase in ISG was greater in the primiparous cows when compared with the multiparous cows. Furthermore, the increase in ISG was markedly greater in pregnant heifers (Exp. 3B) when compared with pregnant cows (Exp. 3A; Table 3).

Using a post-AI sample (sample 2) and a control sample (sample 1) minimally improved the overall utility of the test (Table 4). The ROC curves were slightly improved in primiparous cows when two samples were analyzed (Fig. 3B compared with Fig. 3A) but the overall improvement was appreciably less in heifers (Fig. 3D compared with Fig. 3C). Plasma progesterone concentrations on d 18 after AI was a reasonable test for pregnancy in heifers enrolled in this study (Fig. 3C). A cut-off of 8.2 ng/ml had a true positive rate of 82% and a false positive rate of 0%. Relaxing the cut-off downward increased the true positive rate, reduced the possibility of a false negative, and increased slightly the false positive rate. For example, a cut-off of 7.3 ng/ml had a true positive rate to 94% (6% false negative) and a false positive rate of 14%. The only ideal ROC (100% true positive and 0% true negative) obtained in this study was for heifers when the ratio of sample 2 Oas1 to sample 1 Oas1 was used (Fig. 3D). The cut-off for this ideal test was 1.4 (a heifer with Oas1/Oas1 greater than 1.4 was truly pregnant). Although the results of Exp. 3 showed that using a ratio of sample 2 to sample 1 improves the accuracy of pregnancy diagnosis in heifers this is, of course, an unrealistic sampling scheme for a practical on-farm test. If the test is to be used for its intended purpose (identifying pregnant and non-pregnant cows after insemination) it should have the capacity to do so from a single sample. This level of sensitivity may be beyond what is achievable with an ISG-based test on d 18.

In conclusion, although ISG expression was increased statistically in pregnant cows, the ROC curves derived from the data demonstrated that high false positive rates would be found in lactating cows regardless of the pre-defined cut-off. Multiparous cows would be less likely than primiparous cows to be accurately diagnosed with the ISG test on d 18. Nulliparous heifers appeared to be most-suited for the test given their robust ISG response. Indeed, it appeared that a test based on Oas1 on d 18 could be developed. The cumulative conclusion from this work is that although an ISG-based test could perhaps be used for pregnancy detection after d 20, its use in a program to time AI cows by d 21 where a diagnosis is needed by d 18 may be beyond the achievable sensitivity. Alternative approaches for early pregnancy diagnosis will need to be developed if a d 21 timed re-synchronization is to be enabled. There may be a short period within which the ISG test is viable (after d 20) but the window of utility closes quickly because pregnancy-associated glycoprotein (PAG) testing for bovine pregnancy was highly effective by d 25 after AI (Green et al., 2009).

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Intrapulse changes in progesterone and LH concentrations and luteal blood flow during an estradiol-induced pulse of a metabolite of prostaglandin F2α in heifers

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Prostaglandin F2α

ABSTRACT

Physiologic doses (0.1 mg followed by 0.05 mg 1 h later) of estradiol-17β in sesame-oil vehicle or vehicle alone ($n = 6$/group) were given to heifers on day 14 after ovulation (preluteolysis) to study the effect of estradiol (E2) on circulating 13,14-dihydro-15-keto-PGF2α (PGFM) and on PGFM pulses. Blood samples were collected hourly for 10 h after the 0.1-mg treatment. The E2 group had an increase ($P < 0.03$) in PGFM concentration by 4 h and greater ($P < 0.0001$) prominence of the PGFM pulses. Changes in progesterone and LH concentrations and luteal blood flow within a PGFM pulse were characterized. Within a PGFM pulse in the E2 group, progesterone decreased ($P < 0.04$) between Hours −2 and 0 (ascending portion; Hour 0 = peak of pulse) and increased ($P < 0.04$) between Hours 0 and 2 (descending portion). Intrapulse changes in progesterone were not detected in the vehicle group. Concentration of LH in the E2 group increased ($P < 0.05$) between Hours −1 and 1 and in the vehicle group decreased ($P < 0.03$) between Hours −1 and 0 and increased ($P < 0.06$) between Hours 0 and 1. The percentage of luteal area with blood-flow signals increased ($P < 0.02$) between Hours 0 and 1 in the E2 group, and there were no other differences between hours in either group. Results were consistent with reports of changes in progesterone concentration within a PGFM pulse in cattle and demonstrated intrapulse changes in LH concentration and luteal blood flow during an E2-induced PGFM pulse.

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1. Introduction

Luteolysis in cattle is induced by pulsatile secretion of prostaglandin F2α (PGF) from the endometrium (reviewed in McCracken et al., 1999). The PGF metabolite 13,14-dihydro-15-keto-PGF (PGFM) has been used to represent circulating concentrations of PGF (Kindahl et al., 1976). Regulation of luteolysis in cattle involves endometrial receptors for progesterone, estradiol, and oxytocin (reviewed in Silvia et al., 1991). Early exposure of the uterus to exogenous progesterone shortens the estrous cycle in cattle (Ginther, 1970). Luteolysis also involves circulating estradiol from the developing follicles (Salfen et al., 1999), as demonstrated by follicle destruction or removal of follicle contents (Fogwell et al., 1985; Araujo et al., 2009). A bolus injection of a pharmacological dose of estradiol induces PGF secretion within 6–8 h (Thatcher et al., 1986; Araujo et al., 2009). Administration of estradiol stimulates the release of both uterine PGF and ovarian oxytocin (Al-Matubsi et al., 1997). These and many other studies (reviewed in Silvia et al., 1991) have shown that prior exposure of the uterus to progesterone for about 14 days and short-term exposure to estradiol are components of the mechanism leading to secretion of PGF in cattle.

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Early in spontaneous luteolysis, a decrease in progesterone occurs at the peak of the PGFM pulse followed by an increase or rebound (Ginther et al., 2010a). A single injection of estradiol 14 days after ovulation, but not at 10 days, stimulates a prominent pulse of PGFM with an associated transient depression in progesterone at the peak of the PGFM pulse (Ginther et al., 2010b). Pulses of LH about 4 h in duration stimulate pulses of progesterone during mid-diestrus (Procknor et al., 1986). However, it is not known if a similar temporal relationship between LH and progesterone pulses occurs during spontaneous or induced luteolysis. During spontaneous luteolysis, luteal blood flow increases during the ascending portion of a PGFM pulse, reaches maximum at the peak of the pulse, and remains elevated for 2 h before decreasing (Ginther et al., 2007).

In the present experiment, a physiologic dose of estradiol was used to stimulate a pulse of PGFM so that the temporal associations between the components of a PGFM pulse and the intrapulse changes in circulating progesterone and LH concentrations and luteal blood flow could be studied.

2. Materials and methods

Holstein heifers aged 17–20 mo were used during October in the northern hemisphere. The animals were maintained by *ad libitum* access to a mixture of alfalfa and grass hay, water, and trace-mineralized salt. The day of ovulation was determined by transrectal ultrasonic examinations (Ginther, 1998). All animals remained healthy and in good body condition throughout the studies. The animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

The experiment was done 14 days after ovulation when heifers were expected to be in preluteolysis; luteolysis is reported to begin at 15, 16, or 17 days after ovulation (Ginther et al., 2007). A vehicle group and an estradiol-17β (E2) group were used ($n = 6$ heifers/group). E2 was prepared in a sesame-oil vehicle as described (Ginther et al., 2000). A physiologic dose (0.1 mg) of E2 was given intramuscularly at the first hour of treatment. Dose was based on the report that 0.1 mg will induce a PGFM pulse on day 14 (Ginther et al., 2010b) and on titration studies in heifers that were similar in body weight and age to those in the present experiments (Ginther et al., 2000). In addition, a dose of 0.05 mg was given the next hour and was expected to maintain maximum E2 concentration an hour longer than expected for a single treatment (Ginther et al., 2000). This was done to determine if two treatments would increase the synchrony of PGFM pulses; synchrony was not obtained with a single treatment (Ginther et al., 2010b). An indwelling catheter was inserted into a jugular vein 15 h before treatment and secured in place. Blood samples were collected hourly for 10 h beginning at the first hour of treatment for assay of PGFM, progesterone, and LH.

A transient PGFM fluctuation with a total of at least four values with a progressive increase on the ascending portion and progressive decrease on the descending portion, including the nadirs and peak, was statistically defined as a PGFM pulse as described (Ginther et al., 2007). When the coefficient of variation (CV) of the values composing the ascending portion, peak, and descending portion of the transient fluctuation was at least three times greater than the mean intra-assay CV, the fluctuation was defined as a PGFM pulse. The hour of the peak of CV-identified PGFM pulses (Hour 0) was used for centralization and intrapulse evaluation of changes in progesterone and LH concentrations and the extent of luteal blood flow between Hours −2 and 2. This 5-h interval was used in the analyses because the pulses in the vehicle group did not include Hours −3 and 3.

Percentage of area of the corpus luteum with color-Doppler signals of blood flow was determined for each hour as described (Ginther et al., 2007). Briefly, the color-Doppler transducer was passed transrectally several times over the entire corpus luteum, and the real-time images were stored on film. After PGFM pulses were located by PGFM assay, the film clips for the hours of each PGFM pulse were coded randomly by Operator 2, so that Operator 1 was unaware of the source of the film clips. An estimate of the percentage of luteal area with color-Doppler signals of flowing blood (≥6 cm/s) was made by Operator 1 as described (Araujo and Ginther, 2009).

Blood samples were collected into heparinized tubes and immediately placed in ice water for 10 min before centrifuging ($2000 \times g$ for 10 min). Plasma samples were assayed for PGFM by an enzyme immunoassay that was developed and validated in our laboratory for use in bovine plasma (Ginther et al., 2010a). The intra-assay and interassay CVs and sensitivity were 14.3%, 21.9%, and 4.4 pg/ml, respectively. Plasma progesterone concentrations were measured using a solid-phase radioimmunoassay kit containing antibody-coated tubes and 125I-labeled progesterone (Coat-A-Count Progesterone: Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described for bovine plasma in our laboratory (Ginther et al., 2007). The intra-assay CV and sensitivity were 7.2% and 0.02 ng/ml, respectively. Plasma concentrations of LH were measured by radioimmunoassay as described and validated in our laboratory (Palhao et al., 2009). The intra-assay CV and sensitivity were 6.2% and 0.04 ng/ml, respectively.

Data that were not normally distributed were transformed to natural logarithms or ranks. Concentrations of PGFM were analyzed for the main effect of hour by the SAS MIXED procedure (Version 9.2; SAS Institute, Inc., Cary, NC, USA), using a REPEATED statement to account for autocorrelation between sequential measurements. For study of changes in progesterone, LH, and blood flow within the hours of a PGFM pulse, data were centralized to the peak of the PGFM pulse (Hour 0), and comparisons among hours were made by paired *t*-tests. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± S.E.M.

3. Results

Mean hourly concentrations of PGFM for the 10-h sessions of hourly sampling showed main effects of group ($P < 0.002$) and hour ($P < 0.01$) and an interaction of group

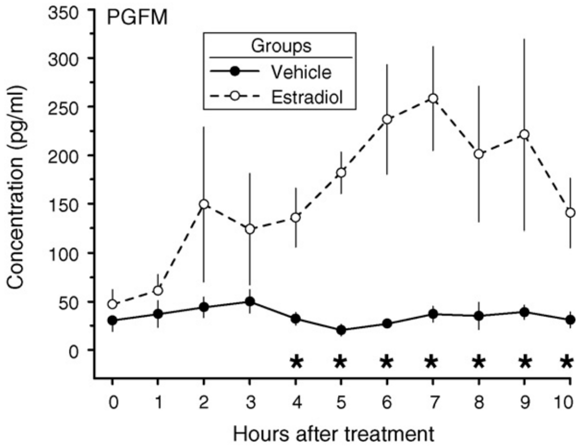

Fig. 1. Mean ± S.E.M. for circulating concentrations of PGFM after treatment with estradiol or vehicle on day 14 after ovulation ($n = 6$ heifers/group). The interaction of group and hour was significant. The asterisks indicate hours of a significant difference between groups.

and hour ($P < 0.006$; Fig. 1). Concentrations of PGFM for the vehicle group did not differ significantly during the 10 h. Concentrations were different ($P < 0.003$) among hours for the E2 group with the first significant increase ($P < 0.03$) at 4 h posttreatment. The concentrations were greater ($P < 0.05$) in the E2 group than in the vehicle group at 4–10 h. There were no differences in hourly mean concentrations of progesterone (range of means, 7.0–9.1 ng/ml) during the 10 h within and between the two groups (data not shown).

Pulses of PGFM were identified statistically in 4 of 6 heifers in the vehicle group ($n = 6$ pulses) and in 6 of 6 heifers in the E2 group ($n = 8$ pulses). There was a group effect ($P < 0.0001$), hour effect ($P < 0.0001$), and interaction of group and hour ($P < 0.008$) for PGFM pulses. Concentration of PGFM was greater ($P < 0.02$) in the E2 group at each hour of a pulse (Fig. 2). The interaction reflected primarily greater differences between groups at Hour 0 (E2 compared to the vehicle group: 315 compared to 64 pg/ml) and Hour 1 (260 compared to 28 pg/ml) than at the remaining hours. For progesterone centralized to the peak of a PGFM pulse (Hour 0), concentration was not different among Hours −2 to 2 in the vehicle group. In the E2 group, progesterone concentration decreased ($P < 0.04$) between Hours −2 and 0 and increased ($P < 0.04$) between Hours 0 and 2. Concentration was less ($P < 0.05$) at Hour 0 in the E2 group than in the vehicle group. Concentration of LH in the vehicle group was greater at Hour −1 than at Hour 0 ($P < 0.03$) and less at Hour 0 than at Hour 1 (approached significance, $P < 0.06$). In the E2 group, the LH concentration decreased ($P < 0.05$) between Hours −2 and −1 and increased ($P < 0.02$) between Hours −1 and 1. Percentage of luteal area with blood-flow signals centralized to the PGFM peak increased ($P < 0.02$) between Hours 0 and 1 in the E2 group. There were no other significant changes in blood flow for either group.

4. Discussion

Direct support that the experiment was conducted before the beginning of luteolysis was the finding in the vehicle group that progesterone concentration did not

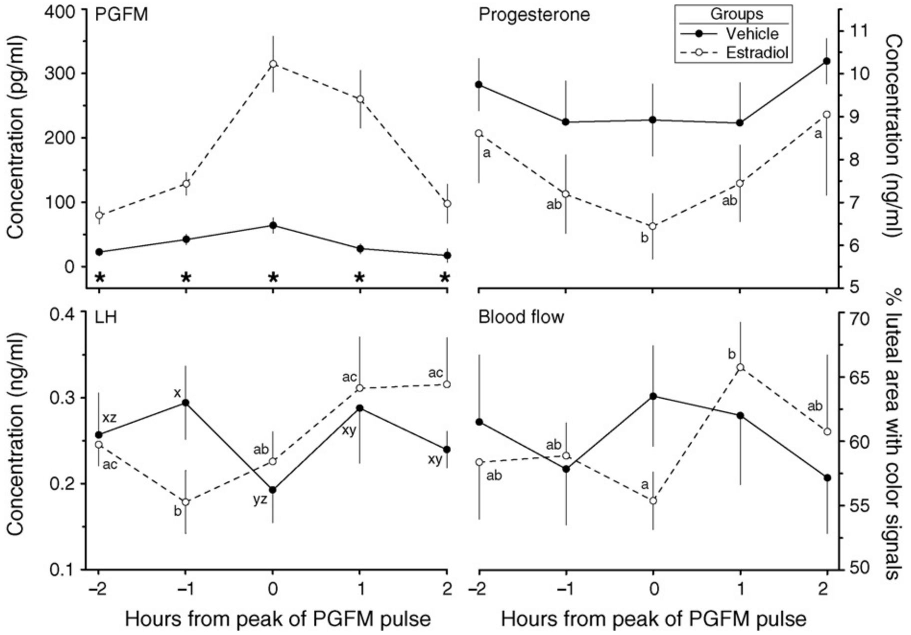

Fig. 2. Mean ± S.E.M. intrapulse concentrations of PGFM, progesterone, and LH and percentage of luteal area with signals of blood flow normalized to peaks of PGFM pulses after treatment with estradiol or vehicle on day 14 ($n = 6$ heifers/group). Hours of a difference ($P < 0.05$) in PGFM pulses between groups are indicated by asterisks. Progesterone concentrations within a PGFM pulse did not change in the vehicle group and differences between hours in the estradiol group are indicated. Concentrations of LH changed within each group between hours of a PGFM pulse as indicated. Percentage of luteal area with blood-flow signals increased between Hours 0 and 1 in the E2 group with no other detected changes in either group. abc = progesterone, LH, and blood-flow means with different letters in the estradiol group are different ($P < 0.05$). xyz = LH means with different letters in the vehicle group are different ($P < 0.05$) and the difference between Hours 0 and 1 approached significance ($P < 0.06$).

change significantly during the 10-h sessions. The absence of a progesterone response in the vehicle group was likely a consequence of the non-prominent PGFM pulses. The increase in PGFM concentrations after treatment with E2 is consistent with previous reports (Thatcher et al., 1986; Ginther et al., 2010b).

Stimulation of PGF secretion by E2 treatment was indicated also by greater prominence of the PGFM pulses in the E2 group, confirming a previous report (Ginther et al., 2010b). Each pulse of PGFM in the E2 group was greater than for the most prominent pulse in the vehicle group. In addition, a PGFM pulse was identified in only 4 of 6 heifers in the vehicle group. In the E2 group, a prominent PGFM pulse was induced in each heifer, and the peak of the first pulse in the six heifers occurred at 2, 3, 4, 5, 6, and 7 h after treatment. This indicated that the two E2 treatments did not induce PGFM pulses that were synchronized adequately for future experimental purposes. Intrapulse changes in progesterone concentrations within an E2-induced PGFM pulse in the preluteolytic period was shown by a decrease during the ascending portion of the pulse and an increase during the descending portion, confirming a previous report (Ginther et al., 2010b). The increase during the descending portion seemed similar to the rebound that occurs 1 or 2 h after the beginning of infusion of PGF to simulate a PGFM pulse (Shrestha et al., 2010a,b).

The increase in LH during Hours −1 to 1 of an induced PGFM pulse and the temporary depression in LH at Hour 0 in the vehicle group are novel findings. These results indicate a need for further study of the temporal and functional interrelationships among PGF, progesterone, and LH before and during spontaneous luteolysis. Although pulses of LH are temporally associated with pulses of progesterone during mid-diestrus (Procknor et al., 1986), a balance between a luteotropic effect of LH and a luteolytic effect of PGF has not been considered as a component of the luteolytic process. An increase in percentage of luteal area with blood-flow signals occurred only in the E2 group and only during the descending portion of a PGFM pulse. Most of the blood-flow increase occurred between Hours 0 and 1 concomitant with the beginning of the progesterone increase. During spontaneous luteolysis, luteal blood flow increases during the ascending portion of a PGFM pulse, reaches maximum at the peak of the pulse, and remains elevated for 2 h before decreasing (Ginther et al., 2007). An increase in luteal blood flow during the hours of an expected or demonstrated progesterone rebound has been reported for exogenous PGF, using several doses, routes, and methods of administration (Ginther et al., 2009; Shrestha et al., 2010a,b). The reported 2-h plateau in blood flow beginning at the PGFM peak in a spontaneous pulse occurred at the time of the progesterone rebound in the present E2-induced PGFM pulses. On a temporal basis, the increase in blood flow as well as the increase in LH or both in the present study may have accounted for the progesterone rebound, and further study is needed.

In conclusion, administration of E2 during two consecutive hours in the preluteolytic period on 14 days after ovulation and collection of blood samples for each hour for 10 h resulted in PGFM pulses that were more prominent in the E2 group than in a vehicle group. Progesterone decreased between Hours −2 and 0 (ascending portion) of a PGFM pulse (Hour 0 = peak of pulse) and increased or rebounded between Hours 0 and 2 (descending portion). Concentrations of LH increased in the E2 group between Hours −1 and 1 and were transiently depressed in the vehicle group at Hour 0. The percentage of area of the corpus luteum with blood-flow signals increased concomitantly with the progesterone rebound. Thus, the E2-induced PGFM pulses during the preluteolytic period were temporally associated with intrapulse changes in progesterone and LH concentrations and luteal blood flow.

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The effect of offering an energy and protein supplement to grazing canchim beef cows either postpartum or both pre- and postpartum on lipid blood metabolites and folliculogenesis

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ABSTRACT

This study was designed to evaluate the effect of nutritional supplementation offered during the pre- and postpartum periods on serum cholesterol, triglycerides and total lipids of Canchim beef cows and their relationship with folliculogenesis. Thirty cows with predicted calving date between September and October, kept in pastures of *Brachiaria brizantha* cv. Marandú together with their calves, were randomly distributed into three experimental groups: the first received only a mineral mixture (Control Group, CG); the second group received a concentrate with 16% crude protein/kg dry matter (DM) and 3000 kcal digestible energy/kg DM offered for 45 days prepartum and 120 days postpartum (PREG); the third group received the concentrate from parturition until the 120th day postpartum (POSG). Consumption was estimated at 1% of body weight, and each cow received approximately 4.0 kg/day (fresh weight) of supplement. Blood samples were taken and an ultrasound examination of the ovaries was performed twice a week until the 60th day postpartum. The body condition score (BCS) and the weight of the cows were recorded at 15-day intervals from calving until the 60th day postpartum. Data are presented as mean ± SEM. Mean weight and BCS at calving were, respectively, 448 ± 54.9 kg and 6.2 ± 0.25 (PREG); 432 ± 71.1 kg and 5.5 ± 0.69 (POSG); and 434 ± 66.4 kg and 5.5 ± 0.69 (CG). Total cholesterol (TC), triglycerides (TRIG) and total lipids (TLIP) were measured using colorimetry until the 60th day postpartum. TC averages were PREG 186 ± 62.6 mg/dL, POSG 159 ± 43.1 mg/dL and CG 133 ± 35.1 mg/dL ($P < 0.05$). For TRIG, the means were PREG 29 ± 11.3 mg/dL ($P < 0.05$), POSG 24 ± 8.1 mg/dL and CG 26 ± 12.1 mg/dL ($P > 0.05$). Serum concentrations of TLIP were PREG 588 ± 145.6 mg/dL, POSG 512 ± 137.6 mg/dL and CG 452 ± 122.4 mg/dL ($P < 0.05$). The first dominant follicle (DF) was identified on Day 21 ± 10.3 (PREG), 36 ± 28.5 (POSG) and 51 ± 32.8 (CG) after calving. The difference between PREG and CG was significant ($P < 0.05$). TC was positively correlated with the calving to first estrus interval ($P < 0.05$). Results showed that nutritional supplementation before parturition assured good body condition at calving and suggested that it was effective at increasing cholesterol availability to maintain ovarian follicle function and to favor earlier resumption of ovarian activity.

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1. Introduction

Reproductive efficiency affects productive performance and most of the reproductive failures in beef cattle are

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attributed to inadequate nutrition. In order to achieve maximum reproductive efficiency in a herd, physiological, nutritional and management factors must be perfectly integrated, adapted to the prevailing physical conditions and, most importantly, fully functional. Upon reviewing the literature on reproductive physiology, Malven (1984) concluded that the resumption of pituitary activity, uterine involution and cessation of the inhibition of pulsatile LH secretion caused by lactation, follicular development, estrus and an adequate luteinic function are necessary for resumption of the reproductive function in the postpartum period. The reestablishment of postpartum ovarian cyclicity and the return to estrus depends on how the hypothalamus, hypophysis and ovaries work together.

Energy balance, which is perceived by the reproductive axis as a variety of nutritionally induced cues, has a profound effect on the postpartum anoestrus interval in beef cows (Richards et al., 1986; Sinclair et al., 2002; Hess et al., 2005) and dairy cows (Beam and Butler, 1997). The prepartum nutritional level is the most important factor for decreasing the interval between parturition and first ovulation (Short and Adams, 1988). Nutritional deficiencies present at the time of parturition are more important than those appearing after parturition, especially because they prolong the anoestrus period. According to McSweeney et al. (1993), weight gain and body condition during the peripartum period and throughout the first two months is extremely important to decrease postpartum anestrus in *Bos taurus indicus*. The duration of the calving to first estrus interval was greater for beef cows fed to calving in thin body condition, and both postpartum protein intake and body condition at calving influenced the size of the dominant follicle at the first postpartum estrus (Lents et al., 2008).

Antral follicle growth in cattle occurs in wave-like patterns during the ovarian cycle and postpartum anoestrus period (Ginther, 2000). Postpartum follicular development involves the growth and regression of follicles smaller than 8 mm up to the detection of the first dominant follicle, which takes place between 10 and 15 days postpartum (Murphy et al., 1990; Savio et al., 1990; Stagg et al., 1995). By using ultrasound to assess follicular development, Ryan et al. (1994) found that 17 days after parturition cows with a good body condition during parturition had a larger number of medium-sized (5–9 mm) and large follicles (≥10 mm) relative to those cows whose body condition was low at parturition. Failure of ovulation rather than lack of dominant follicle development is responsible for the prolonged postpartum anoestrus interval (Diskin et al., 2003).

Plasma levels of blood metabolites have been used to demonstrate their effect on the reproductive physiology of cows. Nutritional supplementation can change the pattern of these metabolites and some hormones (Oliveira Filho et al., 2000; Ruas et al., 2000). Cholesterol, triglycerides and low- and high-density lipoproteins are metabolites that are likely to indicate changes in the lipid and energetic metabolism (Mancio et al., 1999). Williams (1989) described a linear increase in blood concentration of cholesterol and triglycerides during the first 8 weeks after parturition. Factors such as the type of diet, age, breed, reproductive status and lactational status can affect both the content and the composition of circulating lipids (Bitman et al., 1990). Diets rich in lipids increase serum concentrations of cholesterol (Ryan et al., 1992; Lammoglia et al., 1996; Mancio et al., 1999). Westwood et al. (2002) demonstrated the importance of plasmatic cholesterol concentrations in the reproductive function of cows and associated them with the return of estrus, the parturition-conception interval, conception and pregnancy rates. On the other hand, Ciccioli et al. (2003) reported no alterations in glucose, NEFA, IGF-I, leptin, insulin or thyroxine in the plasma of beef cows maintained on native pasture and supplemented with 0.9 or 1.8 kg/day of a 38% CP soybean meal-based supplement during the postpartum period, concluding that they may not be limiting factors for the first estrus after calving, but cows that maintain or lose body condition during lactation have a prolonged calving to first estrus interval.

The aim of this study was to characterize blood cholesterol, triglycerides and total lipid patterns during the postpartum period, and associate them with follicular development, in Canchim cows receiving nutritional supplementation before and after parturition.

2. Material and methods

Thirty Canchim cows (5/8 Charolais × 3/8 Zebu), ranging in age from 4 to 8 years old were used in this study. Cows were stratified based on expected calving date and were randomly assigned to one of the three nutritional regimens (10 cows per treatment): the control group (CG), without supplementation, in which the animals received only mineral supplementation; prepartum (PREG) supplementation, and postpartum (POSG) supplementation. The PREG received nutritional supplementation beginning approximately 45 days before average expected calving and continued receiving a supplement for up to 120 days afterwards. The POSG were supplemented from the time of parturition to 120 days postpartum. After parturition, all the cows were suckled by their calves.

The animals were maintained on a pasture with *Brachiaria brizantha* cv. Marandú, with water *ad libitum*. Although forage availability was not measured, visual evaluation of pastures indicated that there was ample dormant forage during the months of September and October, at the beginning of the experiment, and green forage from November through February. The PREG and POSG were supplemented with a soybean meal-based supplement containing 3000 kcal of digestible energy/kg, 16% crude protein and 85.4% TDN on a dry matter basis (Table 1). Consumption was estimated at 1% of body weight, and the PREG and POSG were fed approximately 4.0 kg/cow/day fresh weight of the supplement. The groups were grazed in separate paddocks, each paddock having an area of approximately 10 ha, and the supplement was offered once daily at 7:00 a.m. Cows in the CG received no supplemental feed. The animals were weighed on the day of parturition and their body condition score (BCS) was visually assessed according to Herd and Sprot (1986), using a scale from 1 to 9 (1 = very thin and 9 = very fat). This procedure was repeated every 15 days until estrus was observed.

Table 1
Composition of the energy and protein supplement (% fresh weight) offered to Canchim beef cows during both pre- and postpartum (PREG) or only in the postpartum period (POSG).

Ingredient composition	%
Corn grain, cracked	67.898
Rice meal	15.000
Soybean meal	15.000
Urea	1.000
Limestone	1.000
Sulphur	0.100
Vitamin A	0.001
Vitamin E	0.001

Ultrasound examination of ovaries and blood samples were performed twice a week, in the morning. An Aloka 210 equipment with endorectal 5 MHz transducer was used to obtain the ultrasound images to monitor follicular development. Follicles were identified at ≥4 mm. Each ovary was located and the image was frozen for the purpose of measuring the follicles, classified as small (4–6 mm), medium (7–9 mm) or large (≥10 mm). Blood was collected from the coccygeal vein and after clot retraction, serum aliquots were kept at −20 °C, until the samples were processed for total cholesterol (TC), triglycerides (TRIG) and total lipids (TLIP) measurements using colorimetry, and a set of commercially available reagents (Doles®).

The recovery of blood samples and other experimental procedures began at calving for all treatments and were continued until the 60th day postpartum, but reliable images of the ovaries were only possible starting from Day 8 after parturition. Monitoring for the first estrus was performed until 180 days postpartum.

Statistical analysis was performed by a computational package (SAS, 2000) using the UNIVARIATE procedure to test the normality of variables. Since there was no normal distribution, the analysis was non-parametric using the procedure NPAR1WAY to perform the Kruskal–Wallis *H* test. To study the strength of association between TC, TRIG, TLIP and the calving to first estrus interval, the Pearson correlation coefficient was calculated using the software WinSTAT® version 2009.1 (Fitch, 2009).

3. Results

Mean weight and BCS at calving were, respectively, 448 ± 54.9 kg and 6.2 ± 0.2 (PREG); 432 ± 71.1 kg and 5.5 ± 0.7 (POSG); and 434 ± 66.4 kg and 5.5 ± 0.7 (CG). Variation in BCS during the period of the study is shown in Fig. 1A. In the first 15 days after parturition, CG and PREG groups showed a decrease in BCS, and then showed little variation until the end of experiment. POSG did not show a decrease in BCS during the first 15 days postpartum.

TC averages were PREG 186 ± 62.6 mg/dL, POSG 159 ± 43.1 mg/dL and CG 133 ± 35.1 mg/dL ($P < 0.05$; Table 2). The concentration of TC was at its lowest level at calving time, and showed an increase during the postpartum period. The CG group presented the lowest values during the experimental period (Fig. 1B)

For TRIG, means were PREG 29 ± 11.3 mg/dL ($P < 0.05$), POSG 24 ± 8.1 mg/dL and CG 26 ± 12.1 mg/dL ($P > 0.05$). The animals in the PREG had the highest values during the peripartum period, 31 ± 7.7 mg/dL, compared to POSG 16 ± 5.5 mg/dL and CG 22 ± 7.3 mg/dL ($P < 0.05$). In that group (PREG), a small decrease was present after calving, while in the POSG, because supplementation began after parturition, a slight increase was verified, reaching 26 ± 5.1 mg/dL by 30 days after calving, remaining stable with a slight tendency to decline afterwards. The same pattern was observed in the CG (Fig. 1B and C).

Table 2
Serum concentration (mean ± SEM) of cholesterol, triglycerides and total lipids during the first 60 days postpartum for grazing Canchim beef cows that were not supplemented (CG), or supplemented with dietary energy and protein both pre- and postpartum (PREG) or only in the postpartum period (POSG).

	PREG $\mu \pm$ SEM	POSG $\mu \pm$ SEM	CG $\mu \pm$ SEM
Cholesterol (mg/dL)	186 ± 62.6^a	159 ± 43.1^b	133 ± 35.1^c
Triglycerides (mg/dL)	29 ± 11.3^a	24 ± 8.1^b	26 ± 12.1^b
Total lipids (mg/dL)	588 ± 145.6^a	512 ± 137.6^b	452 ± 122.4^c

Values with different superscripts within a row are significantly different ($P < 0.05$).

Serum concentrations of TLIP were PREG 588 ± 145.6 mg/dL, POSG 512 ± 137.6 mg/dL and CG 452 ± 122.4 mg/dL ($P < 0.05$; Table 2). The serum concentration of TLIP increased progressively after calving. The animals from PREG and POSG showed higher values than CG (Fig. 1D).

The first ultrasound images of the ovaries could not be obtained until 8–10 days after calving, as a consequence of the location and the size of the uterus during involution. After this period, it was always possible to identify the ovaries. All the animals demonstrated follicular activity, characterized by the development and regression of small (4–6 mm) and medium-sized (7–9 mm) follicles in a similar way among the three treatments. After a period that varied among the different animals, a follicle started to grow more rapidly than the others, whose growth was slower. Some even regressed. The first dominant follicle, with ≥10 mm in diameter, was identified at 21 ± 10.3, 36 ± 28.5 and 51 ± 32.8 days after calving in the PREG, POSG and CG animals, respectively. The mean time needed for the appearance of a ≥10-mm follicle was statistically significant shorter ($P < 0.05$) in the PREG cows. The nutritional supplement also shortened the time to the appearance of a ≥10 mm follicle in the POSG cows relative to CG cows but not to the same degree and the difference was not significant ($P > 0.05$). Follicular development in all the cows of PREG began within 45 days, and the same happened to eight cows in the POSG and five cows in the CG.

The calving to first estrus interval was different for each of the treatments, PREG (91 ± 49 days), POSG (168 ± 19 days) and CG (165 ± 24 days) ($P < 0.01$; Table 3). Although cows on the PREG treatment had a significantly shorter calving to first estrus interval than those on the other two treatments, the coefficient of variation (CV) for this group (55.6%) was much higher than that for the POSG (11.6%) and CG (14.4%) groups of cows. There was a moderate positive correlation between calving to first estrus interval and TC ($r = 0.44$; $P < 0.05$), but not for TRIG and TLIP.

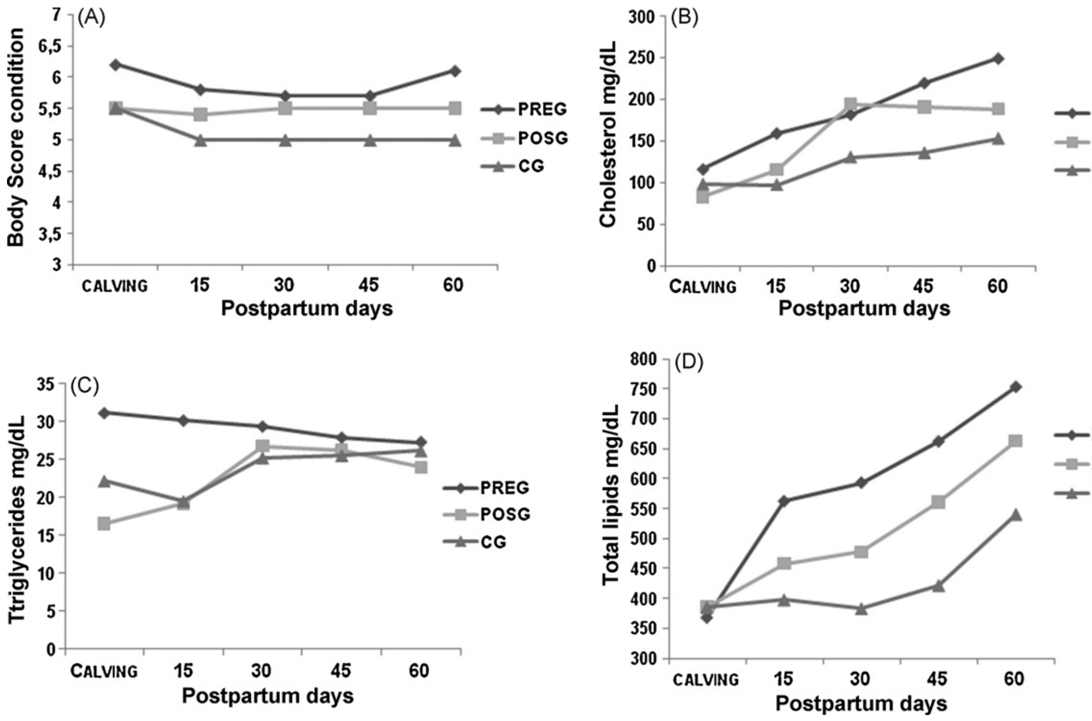

Fig. 1. Body score condition (A), serum total cholesterol (B), serum triglycerides (C) and serum lipids (D) concentration during the first 60 days postpartum for Canchim beef cows that were supplemented with dietary energy and protein both pre- and postpartum (PREG), only in the postpartum period (POSG), or not supplemented (CG).

4. Discussion

TC concentrations determined in this study were higher than those described for cows that received low (87.2 ± 2.1 mg/dL) or high (114.4 ± 2.9 mg/dL) energy (Schrick et al., 1990), and 104.19 mg/dL (Wiley et al., 1991), both studying Angus and Hereford animals. However, they were close to the results described by Godoy et al. (2004), who reported values ranging between 174.22 and 182.71 mg/dL, at 112 days postpartum in Guzerá cows, suggesting the influence of the breed on this variate. Hawkins et al. (1995), and Lammoglia et al. (1996,1997) reported a rise in TC concentrations in cows fed a diet rich in fat. Thomas et al. (1997), using animal fat, soybean and fish oil in the formulation of the animal feed given to Brahman × Hereford cows, demonstrated significant increases ($P < 0.05$) in the blood concentration of TC. Serum concentration of TC in animals receiving supplementation was 1.16 (PREG) and 1.12 (POSG) times higher than the values observed for the animals in the CG, showing that the results were a consequence of supplementation, and corroborating data from Williams (1989), who recorded a 1.7 times increase in the TC levels of animals fed a diet rich in lipids (30% cotton seed), compared to those that did not receive any supplementation.

Around the time of calving, the concentration of TC is at its lowest level and shows a linear increase of approximately 2.2 times throughout the postpartum period in the three groups. The animals in the CG presented the lowest values, as can be seen in Fig. 1A. This linear increase in the postpartum period has been described by several authors in dairy cows (Leroy et al., 2004) and in beef cows, such as Williams (1989) studying Brahman animals, Guedón et al. (1999) in Limousin cows, while Ruas et al. (2000) and Godoy et al. (2004) described this increase in zebu animals.

In this study, lower values for TRIG were observed relative to those reported by Williams (1989), which were between 55 and 75 mg/dL; by Wehrman et al. (1991), 45.1 ± 1.2 mg/dL; and by Thomas et al. (1997), between 45 and 50 mg/dL in Brahman cows; as well as those described by Guedón et al. (1999), 30–40 mg/dL, in Limousin cows. However, a rise in serum concentration of TRIG became evident. The animals in the PREG had the highest values during the peripartum period, 31 ± 7.7 mg/dL, compared to 16 ± 5.5 and 22 ± 7.3 mg/dL for the POSG and CG, respectively. In that group (PREG), a small decrease was present after calving, while in the POSG, because supplementation began after parturition, a slight increase was observed, and reached 26 ± 5.1 mg/dL by 30 days after calving, remaining stable with a slight tendency to decline afterwards. The

Table 3
Calving-first estrus interval (mean ± SEM) for grazing Canchim beef cows that were not supplemented (CG), or supplemented with dietary energy and protein both pre- and postpartum (PREG) or only in the postpartum period (POSG).

Treatment groups	$\mu \pm$ SEM	CV (%)
PREG	90.7 ± 49.5^b	54.6
POSG	167.7 ± 19.4^a	11.5
CG	164.8 ± 23.7^a	14.4

Values with different superscripts within a row are significantly different ($P < 0.05$).

same pattern is observed in the CG (Fig. 1B). This fact was reported by Williams (1989), Wehrman et al. (1991) and Thomas et al. (1997) after supplementation with lipid-rich diets. Ryan et al. (1994) showed that cows with a body condition between 6 and 8 at calving had higher concentrations when compared to those with a body condition 3 or 4. However, Lammoglia et al. (1996) did not observe any changes in the concentration of TRIG after offering a diet containing 3.74%, 5.20% and 6.55% of fat. In the CG, a slight rise in TRIG concentrations until 60 days postpartum was observed. These results agreed with reports by Williams (1989) and Ryan et al. (1994) that observed progressive increases from calving until the eighth week afterwards. Guedón et al. (1999) reported a decrease after parturition, when TRIG levels remained stabilized, while Leroy et al. (2004) have mentioned that following parturition of dairy cows, the TRIG concentration remained relatively low in serum while the TC concentration doubled. This observation is partially caused by the mammary conversion of TRIG-rich lipoproteins (β-lipoproteins or very low and low-density lipoproteins, LDLs) to TC-rich lipoproteins (β-lipoproteins or high-density lipoproteins, HDLs).

A progressive rise in the concentration of TLIP was observed in the three groups after calving (Fig. 1C), with higher values for animals in the supplemented groups, relative to the control group. These results agree with the normal values described by Medway et al. (1973), who reported variations between 200 and 800 mg/dL. They were, however, higher than the mean concentration of 206.3 mg/dL described by Bitman et al. (1990) in six Holstein cows, in which values ranged between 154.2 and 236.9 mg/dL.

According to Murphy et al. (1990), in most lactating beef cows with a reasonable body condition, follicular development begins soon after parturition. No difference ($P > 0.05$) was found regarding the number of follicles, PREG 7 ± 3.4, POSG 6 ± 2.9 and CG 7 ± 3.5, showing that their functional characteristics are the most important factor, rather than the number of follicles.

The relation between weight loss and the consequent change in metabolic variate (cholesterol, triglycerides, glucose and urea) during the peripartum period and the beginning of the lactation period provide information related to reproductive performance and postpartum health (Kim and Suh, 2003). Results of the present study showed that nutritional supplementation before parturition was efficient in ensuring good BCS at calving and in bringing about folliculogenesis earlier, since the first dominant follicle was identified sooner after calving in the PREG animals (21 ± 10.3 days; $P < 0.05$) than in the other two groups (POSG 36 ± 28.5 and CG 51 ± 32.8 days), probably as a consequence of a higher blood concentration of TC, TRIG and TLIP in this group. The faster follicular development occurs the higher the chances of early conception after calving. Guedón et al. (1999) showed that the high serum concentration of cholesterol at the end of the pregnancy period and during the early postpartum period (2 weeks before and 4 weeks after calving) was associated with early resumption of ovarian activity. Similarly, Westwood et al. (2002) reported that dairy cows with a high cholesterol nadir had 2.3 times greater chance of conceiving within the first 150 days of lactation compared to those cows with a low cholesterol nadir. When the plasma cholesterol nadir during the first 10 weeks of lactation was >1.8 nmol/L in that study, the chance of becoming pregnant during the first 150 days of lactation was 2.7 times greater than when nadir cholesterol values were less than 1.8 nmol/L.

Nutritional controls on beef cow reproduction are not mediated by a single nutrient, metabolite, or hormone. A multitude of factors must be considered to understand the complex coordination of the nutrition–reproduction interface. The mechanism through which cholesterol can influence fertility is not fully understood, and may be associated with an improvement in the energy balance rather than with a direct relationship to cholesterol levels. Grummer and Carrol (1988) explained that cholesterol utilized for steroid synthesis by ovarian tissue may be derived from *de novo* synthesis or cellular uptake of lipoprotein cholesterol. The majority of blood cholesterol is transported by either low (LDL) or high (HDL) density lipoproteins, depending on the animal species. Prior to vascularization, only HDL are in follicular fluid and contribute sterol to granulosa cells because other lipoproteins are unable to cross the basement membrane due to their molecular masses. Following vascularization, both LDL and HDL bathe luteal cells. Williams and Stanko (1999) concluded that hyperlipidemia/hypercholesterolemia either changed the rate of progesterone synthesis by the corpus luteum or changed its metabolism or clearance rate.

Reist et al. (2003) reported that cholesterol may be involved in steroid synthesis in the ovary and luteal function, and might thus strongly influence the interval from calving to conception. *In vitro* studies carried out by Gwynne and Strauss (1982) showed that cholesterol performs a regulatory function in steroid production by ovarian tissue. Its use for steroidogenesis affects the estradiol–progesterone relationship in the follicular tissue, which is associated with follicular dominance (Rhodes et al., 2001). Steroidogenic cells sequester cholesterol from circulation by endocytosis and selective uptake, via the membrane receptors found in the granulosa and theca cells throughout the whole follicular development process (Landschulz et al., 1996; Argov et al., 2004), promoting an increase in the expression of these receptors by the mRNA as well as an increase in follicular size (Vassiliou et al., 2001). The cholesterol that has been taken up by plasma is transported to the internal mitochondrial membrane of the ovarian cells and is cleaved by cytochrome P450. Thus, steroid hormone production is dependent on the availability of cholesterol to mitochondrial enzymes (Argov et al., 2004). These authors report that the concentration of cholesterol in the follicular fluid was higher ($P < 0.008$) in the antral follicles that are ≥10 mm, than in <8-mm follicles, with a positive correlation with size.

Changes in cholesterol supply to follicular cells can affect steroidogenesis and, subsequently, ovarian function. A study carried out by Argov et al. (2005) with cows who were subjected to thermal stress showed that a compromised follicular activity was not a consequence of a reduction in aromatase activity for the production of estradiol, but of changes that had taken place in a previous stage of steroidogenesis, which can originate in the expression pattern of mRNA of the cholesterol receptors in the ovarian cells.

Improvement of reproductive performance is often limited by prolonged periods after calving when cows do not display estrous behavior or ovulation. Nutritional management has an important influence on the reproductive activity of postpartum cows, and it could be observed in the PREG cows, which presented the first postpartum estrus earlier than the others did. First postpartum ovulation in beef cows is typically not accompanied by estrous behavior and is followed by a shorter luteal phase than normal (Murphy et al., 1990; Werth et al., 1996). In the current study, PREG cows showed the first estrus around the 60th day postpartum. These cows began to show dominant follicles (≥10 mm) in the third week after calving, suggesting the occurrence of estrus without typical behavior. According Murphy et al. (1990), follicular development and formation of a dominant follicle occur early after calving, but the ovulation rate of this first dominant follicle is low. PREG cows calved in better body condition, with a slight loss until 45 days postpartum, when they were able to recover their body condition at calving (Fig. 1A), indicating that the improvement of body condition at calving through the nutritional supplementation before parturition had a positive effect on the follicular development during the postpartum period.

The positive association between cholesterol concentration and the calving to first estrus interval probably reflects the effect of the increase in bovine lipoproteins fractions involved in the follicular development. Modifications in lipid metabolism during the puerperium seem to be related to resumption of cyclicity during the early postpartum period, since higher serum cholesterol values were observed until 4 weeks after calving in beef cows with early rather than medium and late resumption of ovarian cyclicity (Guedón et al., 1999).

The results of this study show that a good body condition score at calving induced, if necessary, by prepartum energy and protein diet supplementation promotes a high serum concentration of cholesterol, triglycerides and total lipids and is associated with increased follicular development and early resumption of ovarian activity in Canchim beef cows.

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Using a whole farm model to determine the impacts of mating management on the profitability of pasture-based dairy farms

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ABSTRACT

An approach to assessing likely impacts of altering reproductive performance on productivity and profitability in pasture-based dairy farms is described. The basis is the development of a whole farm model (WFM) that simulates the entire farm system and holistically links multiple physical performance factors to profitability. The WFM consists of a framework that links a mechanistic cow model, a pasture model, a crop model, management policies and climate. It simulates individual cows and paddocks, and runs on a day time-step. The WFM was upgraded to include reproductive modeling capability using reference tables and empirical equations describing published relationships between cow factors, physiology and mating management. It predicts reproductive status at any time point for individual cows within a modeled herd. The performance of six commercial pasture-based dairy farms was simulated for the period of 12 months beginning 1 June 2005 (05/06 year) to evaluate the accuracy of the model by comparison with actual outcomes. The model predicted most key performance indicators within an acceptable range of error (residual < 10% of observed). The evaluated WFM was then used for the six farms to estimate the profitability of changes in farm “set-up” (farm conditions at the start of the farming year on 1 June) and mating management from 05/06 to 06/07 year. Among the six farms simulated, the 4-week calving rate emerged as an important set-up factor influencing profitability, while reproductive performance during natural bull mating was identified as an area with the greatest opportunity for improvement. The WFM presents utility to explore alternative management strategies to predict likely outcomes to proposed changes to a pasture-based farm system.

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1. Introduction

High levels of reproductive performance are required in seasonal dairying where inter-calving intervals are constrained to 365 days (Macmillan, 2002a). Mating management is central to reproductive performance of seasonally mated dairy cattle herds. It consists of a series of well-planned management activities requiring astute timing, clear animal identification, accurate record keeping and attention to detail (Macmillan, 2002b). The economic justification for achieving these requirements is often lacking however; which hampers farmers (and consultants) to prioritize the management areas for improvement and allocating the appropriate resources to achieve such improvement. This lack of prioritization is likely because the description of the links between reproductive performance, production and profitability, are limited in large part by the complexity of farm systems. This complexity would include limited understanding of the nutritional influences on fertility and the resilience of milk production to variance in reproductive performance. Furthermore, the costs or benefits of improved reproductive performance are

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often realized over several seasons. These complexities and the confounding effects of "set-up" (farm conditions at the start of the farming year) and weather (demanding flexibility in tactical decision-making) make it virtually impossible for a farmer to improve any aspect of mating management from 1 year to the next and be able to quantify the impact of this change on operating profit.

Modeling is the most feasible approach to linking variances in reproductive performance to production and profitability. Accurately modeled predictions could assist with the development and extension of advice for farmers wishing to improve reproductive performance in a profitable manner. The importance of economic considerations to farmer decision-making on dairy cow reproduction management has previously been recognized in the United States (De Vries, 2006). Within the context of a non-seasonal TMR-diet system, this previous study estimated the value of new and lost pregnancies as US$278 and US$555, respectively.

DairyNZ's whole farm model (WFM) was developed to assist in analyzing and designing farm systems experiments, and to explore various scenarios requiring numerous farm system interactions over multiple years. The model has been described by Wastney et al. (2002) and Beukes et al. (2008). The objectives of this paper were to:

(1) upgrade the existing WFM to include reproductive variables;
(2) evaluate the ability of the model to predict outputs from inputs against observed data of six commercial farms; and,
(3) evaluate the impact of changes in (i) "set-up" (initial conditions on the farm at the start of a farming year on 1 June) and (ii) mating management decisions from 05/06 to 06/07 year on the operating profit of the same six commercial farms.

2. Materials and methods

2.1. Developing the predictive capabilities of the model

The WFM was upgraded to simulate reproduction using a deterministic approach reflecting previously described physiological events required for postpartum cows to re-establish pregnancy (Fig. 1). Key milestones for individual cows are calving, first estrus, insemination(s), conception and pregnancy maintenance. Each step is influenced by cow and management factors. The upgraded WFM predicts the outcome of the interplay of these factors for each animal, and summarizes these outcomes to derive performance indicators at the herd level (e.g. rates of calving, anestrus, inseminations, conceptions and pregnancies).

2.1.1. Cow factors

When the WFM is initialized, calving dates are either manually ascribed or randomly assigned to each cow to reflect the calving pattern of the modeled herd. For cows retained through successive years, the calving date is 282 days from the previously established pregnancy date. By default, the WFM randomly assigns calving dates to replacement heifers to reflect the previous calving pattern for heifers. Alternatively, calving dates can be individually specified by the user.

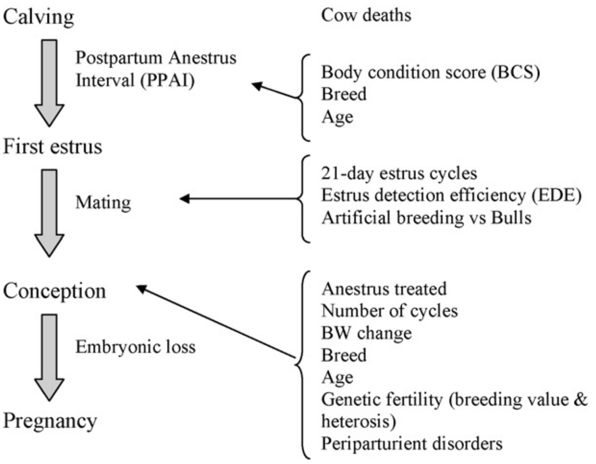

Fig. 1. Physiological events and influencing factors accounted for in the DairyNZ whole farm model (WFM) for re-establishment of pregnancy in the postpartum dairy cow.

The date of first postpartum estrus for every cow is determined by adding a variable postpartum anestrous interval (PPAI in days) to the calving date. The PPAI is selected from a reference table that accounts for the effects of breed and age (Table 1) (Grainger et al., 1982; McDougall et al., 1995; Burke et al., 1995, 2006; Burke and Roche, 2007). The PPAI assigned to individuals is further modified for the effect of body condition score (BCS) at calving (McDougall et al., 1995; Burke et al., 1995), but subject to a minimum value as in Table 1.

$$\mathrm{PPAI} = \mathrm{PPAI}_{\mathrm{reference}} - 8(\mathrm{BCS}_{\mathrm{at\,calving}} - 4.5) \tag{1}$$

where $\mathrm{PPAI}_{\mathrm{reference}}$ is read from Table 1; $\mathrm{BCS}_{\mathrm{at\,calving}}$ is the body condition score of a cow at calving; 8 (days) is the default weighting for the effect of BCS at calving on PPAI (BCS factor in Table 1); 4.5 is regarded as the BCS (on the New Zealand 10-point scale ≈ 2.94 on the US scale; Roche et al., 2004) where there is no modification of the reference PPAI.

Cows have an estrus every 21 days from date of first estrus until pregnancy is established. They are only eligible for insemination when estrus occurs and is detected during defined mating periods specified by the mating policy. Conception rate (CR), defined as the chance of a cow conceiving to an insemination, is assigned to individual insemination events. At any insemination, the CR is initially selected from a reference table (Table 2) that accounts for differences among ages, breeds, whether or not a cow was treated for anestrus, and number of previous estruses before an insemination (Xu and Burton, 2003; McDougall and Compton, 2005). The reference CR is then modified for each individual:

$$\mathrm{CR} = \mathrm{CR}_{\mathrm{reference}} + \text{body weight factor} + \text{fertility factor} + \text{health factor} \tag{2}$$

where $\mathrm{CR}_{\mathrm{reference}}$ from Table 2;

Table 1
Reference postpartum anestrous interval (PPAI) and BCS factor in the DairyNZ whole farm model (WFM) based on Grainger et al. (1982), McDougall et al. (1995) and Burke et al. (2006, 2007). Values presented are the WFM defaults and can be altered by the user.

Breed	From age (year)	To age (year)	Minimum PPAI (days)	Mean PPAI (days)	Standard deviation of PPAI	BCS factor (days)
Holstein-Friesian (HF)	1	<1.5	32	60	5	8
	1.5	<2.5	32	60	5	8
	2.5	<3.5	27	50	5	8
	3.5	<4.5	22	40	5	8
	4.5	99	20	40	5	8
Jersey (J)	1	<1.5	30	55	5	8
	1.5	<2.5	30	55	5	8
	2.5	<3.5	25	45	5	8
	3.5	<4.5	20	35	5	8
	4.5	99	18	35	5	8
HF × J	1	<1.5	17	55	5	8
	1.5	<2.5	17	55	5	8
	2.5	<3.5	25	45	5	8
	3.5	<4.5	20	35	5	8
	4.5	99	18	35	5	8

Body weight factor (%) = $3.0(\Delta BW - 1.5)$ where ΔBW is the percent BW change 3 weeks before the insemination. Maximum positive effect was set at ΔBW 2.8% and maximum negative effect at ΔBW 0.2% (from Roche et al., 2006);

Fertility factor (%) = (effective $BV_{fert} \times 2.4$), where effective BV_{fert} (effective breeding value for fertility) = BV_{fert} + (heterosis factor × 3.5). The heterosis factor depends on the amount of cross-breeding between Friesian (F) and Jersey (J) breed types. The heterosis factor is 1 for F8J8; 5/6 for F6J10/F10J6; 2/3 for F4J12/F12J4; and 0 outside this range. The BV_{fert} is an estimate of an animal's genetic fertility as derived by New Zealand Animal Evaluation Ltd., New Zealand (Harris et al., 2006).

Health factor (%) = −10% for cows with periparturient disorders, and +6.1% for otherwise 'healthy' cows. The reference data in Table 2 were based on a large data set (>100,000 cow-seasons) using re-calving information to confirm previous conception dates, and hence conception rate to inseminations (Xu and Burton, 2003). These data included cows with periparturient disorders and embryonic losses. In the WFM, however, the rates of periparturient disorders and embryonic failure (defined below) are modeled explicitly from user settings (the default is 10% of the herd for each occurrence). It was, therefore, deemed necessary to apply an adjusted CR from the reference CR table to avoid double-counting. Since the reference CR of cows with periparturient disorders is adjusted by −10% (McDougall et al., 2007), the reference CR for healthy cows had to be adjusted +6.1% (+1.1% to compensate for periparturient disorders and an estimated +5% to compensate for the contribution of embryonic failure to final non-pregnant rates) to achieve the "average" CR across a herd with a "normal" prevalence of periparturient disorders and embryonic failure.

For modeling purposes it was assumed that embryonic failure can occur up to 35 days after conception (early embryonic failure). The chance of failure is applied uniformly across all cows and days within this period of gestation, independent of any pre-existing cow conditions. The proportion of conceptions that ultimately fail will approximate the default (i.e. 10%) or alternative user-specified embryonic failure rate. If embryonic failure occurs, the model assumes the cow will return to estrus 21 days later, and can re-establish a pregnancy provided there is a successful insemination within the defined breeding period.

2.1.2. *Management factors*

On the "Management" page of the WFM, the user constructs a mating policy with dates for anestrous treatments (if applied), artificial breeding (AB) and bull mating periods with corresponding levels of estrous detection efficiency (EDE). The EDE is a user-defined variable. It can be set to dif-

Table 2
Reference conception rate (CR) in the DairyNZ whole farm model (WFM) based on Xu and Burton (2003) and McDougall and Compton (2005). Values presented are the WFM defaults and can be altered by the user.

Breed	From age (year)	To age (year)	Conception rate (CR%)			
			Anestrous treated cows (synchronized insemination only)	First postpartum estrus	Second postpartum estrus	Third postpartum estrus (and beyond)
Holstein-Friesian	1	<1.5	40	45	52	62
	1.5	<7.5	40	45	52	62
	7.5	99	40	45	52	62
Jersey	1	<1.5	40	42	49	59
	1.5	<7.5	40	42	49	59
	7.5	99	40	42	49	59

ferent values over different time periods to reflect temporal differences in human capability to detect estrus during the AB period, and the ability of bulls to detect estrus during the natural mating period. The user also specifies the pre-mating EDE which subsequently influences the number and accuracy of treating cows for anestrus.

The mating policy in the WFM includes an option to shorten the PPAI by applying an anestrous treatment to anestrous cows. In this case the user will set a start date for treatment and a subsequent date for the synchronized insemination. Only cows that have calved > 21 days before the herd's planned start of mating date are eligible for treatment. The CR at the first insemination in treated cows is, by default, less than that of spontaneous cycling cows (Table 2; McDougall and Compton, 2005).

Additional settings in the mating policy include death rate at calving (2% default) and dates for pregnancy tests. The pregnancy test policy option ensures total accuracy in identifying non-pregnant cows, whereas visual estimates of estrus by the herd manager can result in non-pregnant cows being inadvertently retained rather than culled before the next calving.

During the AB period, the user can select from a range of bull semen with different genetic traits e.g. milk production, body weight, fertility, and length of gestation. The bull's genetic length of gestation will affect the next calving date, while the other parameters are accounted for in the genetic merit of the female off-spring.

Reproductive outcomes are also affected indirectly by policies dealing with pasture and supplement feeding, milking frequency and drying-off dates since these policies influence calving BCS and pre-mating BW changes, as previously described.

2.1.3. Randomness

The WFM can generate varying outcomes with successive iterations of the model to reflect the inherent randomness of biological systems. The user can influence the PPAI to be assigned to individual cows in one of three ways. The random option will assign PPAI from a normal distribution, with different iterations of the same scenario producing varied results. This means that with every iteration of the same scenario the same cow may be assigned a different random PPAI.

The semi-random option uses the same normal distribution in such a way that individual cows are assigned random PPAI, but with different iterations of the same scenario each cow will be assigned the same PPAI, ultimately producing the same outcomes at herd level.

With the third option (non-random) the mean value in the reference table is assigned to each cow in a particular category.

The random and semi-random options are also available for modifying the assigned CR to a particular insemination. The random CR option will randomly select a value between 0 and 1 and compare it to the chance of CR success assigned to the insemination event. If for example the randomly selected value is 0.75 and the CR assigned to the insemination success is 0.6, the mating event will be unsuccessful. With the semi-random option the assigned CR values are added cumulatively with each new insemination and a cow conceives to an insemination if the addition of her assigned CR value to the cumulative total exceeds units of 1.0 starting with 0.5, that is 0.5, 1.5, 2.5 etc. For example, a sequence of inseminations with assigned CR of 0.6, 0.4, 0.8, 0.5, 0.4 would respectively result in success, failure, success, failure, success. This approached ensured that the final CR for any simulated herd size (even < 10 cows) was as close as possible to the mean CR of the individual cows.

2.2. Evaluating WFM against observed data from commercial dairy farms

Reproductive performance data from six commercial dairy farms for the 05/06 mating season (October 2005–February 2006) were made available (C. W. R. Compton pers. comm., Animal Health Centre, Morrinsville, New Zealand; LIC herd improvement records, Hamilton, New Zealand). Additional information including farm system type, feeding levels, and management policies were collected directly from farmers and rural professionals working with the farmers. This information was used to initialize the WFM to reflect system conditions for each of the six farms examined. The farm systems varied from:

- a high-stocked farm (4 cows/ha) feeding supplements in early (spring) and late lactation (fall) to a mixture of dairy cow breeds (farm A);
- a farm feeding minimal silage in spring and turnips (Brassica) in summer to a mixture of Holstein-Friesian and crossbred cows (3 cows/ha) (farm B);
- a high-input farm with supplements being fed to large crossbred cows all year round (3.8 cows/ha) (farm C);
- a farm with a no-nitrogen fertilizer policy and Jersey cows (4 cows/ha) being milked once-a-day for part of the year to protect their body condition (farm D);
- a farm with Holstein-Friesian cows being fed silage, palm kernel extract and turnips in an effort to maintain BCS on a hilly terrain (2.8 cows/ha) (farm E);
- a farm with small amounts of maize and pasture silage fed to Jersey cows in the fall (4 cows/ha) (farm F).

In the model, all farms had to be scaled down to 100-cow herds to fit the available computing power. The composition of the simulated herds in terms of calving spread, breed and age (%) were reflected in the scaled-down herds because these cow factors are important drivers of model outcomes (see Section 2.1.1). The scenarios were then run using real climate data from the Ruakura Climatological Station (supplied by the National Institute of Weather and Atmosphere, New Zealand) for the 05/06 year (1 June 2005–31 May 2006) to drive the pasture sub-model. Scenarios were run with the semi-random settings for PPAI and CR in the mating policy, resulting in variation among cows, but ensuring a deterministic outcome of the policy on overall herd reproductive performance. It was important to achieve a deterministic outcome to investigate the effects of changes in the system (e.g. set-up and mating management) without having the results being confounded by chance events. Numerous feeding, production and reproduction key performance indicators (KPI) were used to

Table 3

Observed (Obs), predicted (Pred) and difference (Diff% = |Pred–Obs|/Obs × 100) of key performance indicators for six commercial Waikato farms (A–F) for the 05/06 year.

	A			B			C			D			E			F		
	Obs	Pred	Diff%	Obs	Pred	Diff%	Obs	Pred	Diff%	Obs	Pred	Diff%	Obs	Pred	Diff%	Obs	Pred	Diff%
End farm cover (kg DM/ha)[a]	2125	2531	19	2209	2223	1	2073	2273	10	1946	1976	2	2274	2266	0	2226	2351	6
End cow BW (kg)[b]	460	536	17	450	529	18	480	513	7	440	454	3	500	602	20	360	402	12
Maize silage fed (kg DM/cow)				244	223	9	906	925	2	285	287	1				103	98	5
Palm kernel fed (kg DM/cow)	145	143	1				411	458	11				391	337	14			
Grass silage fed (kg DM/cow)	369	342	7	220	232	5	240	231	4	128	138	8	289	247	15	114	144	26
Turnips fed (kg DM/cow)				182	162	11							153	130	15			
Hay fed (kg DM/cow)	40	39	3				23	11	52	38	41	8	66	76	15	65	63	3
Silage made (kg DM/cow)	309	253	18	132	142	8	189	162	14	141	154	9	143	183	28	120	133	11
Milksolids (kg/cow)[c]	317	317	0	335	346	3	455	499	10	355	388	9	316	317	0	308	313	2
Milksolids (kg/ha)	1134	1244	10	1064	1029	3	1863	1899	2	1115	1193	7	912	862	5	1230	1203	2
Anestrous treated (%)	11	22	100	31	23	26	33	34	3	21	33	57	16	20	25	6	17	183
3-week SR (%)[d]	90	90	0	93	95	2	90	93	3	93	87	6	76	78	3	90	87	3
4-week SR (%)	93	96	3	95	95	0	93	96	3	95	96	1	81	84	4	94	92	2
4-week in-calf (%)	64	64	0	64	60	6	61	64	5	62	66	6	44	46	5	58	58	0
8-week in-calf (%)	82	83	1	81	83	2	82	82	0	82	79	4	68	73	7	79	77	3
10-week in-calf (%)	88	90	2	84	85	1	86	87	1	87	87	0	74	77	4	84	85	1
Non-pregnant rate (%)	11	10	9	8	7	13	9	6	33	13	12	8	15	17	13	12	11	8

[a] Average herbage mass at the end of the farming year on 31 May.

[b] Average cow bodyweight at the end of the farming year on 31 May.

[c] Milksolids = fat + protein.

[d] SR = submission rate.

evaluate the model predictions against measures observed on-farm (Table 3). Each of the six scenarios was fine-tuned using cow genetic merit for milk yield, an index of inherent soil fertility in the pasture sub-model, and factors for which little or no information existed, such as EDE, in an attempt to achieve predicted reproductive KPI within 5% of the observed value. A 10% margin was deemed acceptable for the other KPI. EDE was a useful factor for achieving an acceptable fit for the reproductive KPI. However, it was only changed within a realistic range for the AB period (80–95%) and natural mating period (60–90%), and it was only changed when there was justification for the change e.g. information about the experience of the herd manager, presence/absence of heat detection devices, and information about numbers and types of bulls for the natural mating period.

2.3. Modeling the impact of change in farm set-up and mating management

Results of the model evaluation were discussed with the participating farmers and they identified certain areas for improvement, which they implemented in their mating management, and in some cases in their general tactical decision-making for the 06/07 year. Performance monitoring of the six farms continued through the 06/07 year by collecting data on farm physical and reproductive KPI (through farmer interviews and herd improvement records). The actual management changes farmers implemented across the 2 years were grouped into three categories (Table 4). Set-up is a description of the farm conditions at the start of the seasonal farming year (1 June) and together with mating management has a direct bearing on reproductive performance. Seasonal management has an indirect effect on reproductive performance through feeding, and includes the tactical decisions that will be dictated by a given year in an attempt to supply the daily feed demand of the herd. Observed performances in the 06/07 year differed from 05/06 because of differences in farm set-up, mating management and climate, demanding different feeding and other seasonal management decisions.

The upgraded WFM, evaluated for each farm for the 05/06 year, was used to ask the question, "How much of the difference in operating profit from 05/06 to 06/07 can be explained by farm set-up differences and how much can be explained by mating management differences?" A first step was to initialize the WFM for each farm with the observed set-up, mating management and seasonal management for the 06/07 year. The predicted performances from these simulations were the results of interactions between set-up, mating management and seasonal management making it impossible to differentiate between the individual effects of these factors. In an attempt to separate the effects of set-up and mating management, the 06/07 scenario for each farm was used and the set-up for 06/07 replaced by the set-up for 05/06 and simulated. Next, mating management for 06/07 was replaced by mating management for 05/06, and finally, both set-up and mating for 06/07 was replaced by set-up and mating for 05/06. In the model, seasonal management was confounded by

Table 4
Three management categories, and options within these, that were altered within the DairyNZ whole farm model (WFM) to reflect actual farm management and performance levels.

Seasonal management	Reproductive management	
	Set-up	Mating management
Supplements fed	Average herbage mass	Estrous detection efficiency – AB
Dry-off date	Herd BCS	Estrous detection efficiency – bull
Conservation	Herd age profile	Planned start of mating
Topping	Calving spread	Anestrous intervention
Culling	Planned start of calving	
N-fertilizer use		

year, meaning that seasonal management for 06/07 could not be replaced by seasonal management for 05/06 (see Table 5 for a summary of the scenario development process).

2.4. Assumptions and data analysis

Scenarios were simulated over a full year (1 June 2006–31 May 2007) and reports on milk production, herd statistics and economic farm surplus (EFS = operating profit in NZ$/ha) were produced. Default economic input values, derived from the DairyNZ Economic Farm Survey (Dexcel Ltd., 2004), were used to calculate EFS. Two predicted KPI, 6-week in-calf rate and non-pregnant rate, do not have economic consequences in the simulated year, but affect the economics in the following year(s). EFS was, therefore, adjusted to account for these two KPI in the simulated year. Previous modeling estimated the effect of change in 6-week in-calf rate in the following year to be worth approximately $4/cow per percent change. An adjustment factor of $12/ha/percent change at an average stocking rate of 3 cows/ha was used. The $1000 difference in the selling price of a pregnant cow ($1500) compared to a non-pregnant cow ($500) was used to estimate the impact of a change in non-pregnant rate to be worth $15/ha/percent change.

The unique system for each farm precluded using farms as replicates, and therefore, no statistical analysis was performed on the results. For each farm, a change in set-up from 05/06 to 06/07 resulted in two calculated $-values/ha (one with 05/06 mating and one with 06/07 mating). Similarly, a change in mating resulted in two answers (one with the 05/06 set-up and one with the 06/07 set-up). The two answers for each change differed because of interactions, but they were generally within the same order of magnitude. This allowed an investigation of the trends across the six farms regarding key set-up and mating management factors and also an estimation of the magnitude of impact on operating profit.

3. Results

Modeled predictions of feed supplied, milksolids (fat and protein) production and reproduction indicators were generally within 5–10% of the observed for all farms (Table 3). Notable exceptions were "end cow BW" (average bodyweight at the end of the farming year on 31 May) and prevalence of anestrous cow treatments, where the WFM typically over-predicted these values.

Farm E proved the most difficult for the WFM to accurately predict the observed. The modeled amount of feed supplied had to be reduced to match the observed milksolids production for this farm. Despite modeling farm E with reduced feed supply as compared to observed, end cow BW remained substantially over-predicted (Table 3).

Table 6 shows the unique set of conditions for each farm and how these affected changes in profitability across the two seasons.

The later planned start of mating in farm A was beneficial in 06/07 with more cows cyclic at start of mating, leading to a greater 6-week in-calf rate and a lower non-pregnant rate at the end of mating. However, this will also result in a later planned start of calving in 07/08, which may be optimal if cold weather results in slow pasture growth at the start of the year, but it may also reduce days in milk, production and profit in that year. Profit in 06/07 was not adjusted for any of these eventualities.

In farm B the 4-week calving rate improved from 63% to 74%, heifer BCS from 4.5 to 5.5 and cow BCS from 4 to 4.4 resulting in a gain of $229/ha from 05/06 to the 06/07 year. The 2% increase in non-pregnant rate due to the shorter mating in farm B in 06/07 is likely to turn into a gain in the longer term when low-fertility cows are culled, calving spread is compacted and more days in milk are realized.

Farm C demonstrated the importance of stocking rate in high-input systems. The lower stocking rate in 06/07 resulted in poorer pasture utilization and lower production per hectare that was not offset by a similar reduction in input costs.

Table 5
Scenario development for step three of the exercise: modeling the impact of change in farm set-up and mating management. The process was repeated for each of the six farms.

	Set-up year	Mating management year	Seasonal management year
Evaluation scenario representing actual management	05/06	05/06	05/06
Scenario representing actual attempts to improve management	06/07	06/07	06/07
Experimental scenario 1	05/06	06/07	06/07
Experimental scenario 2	06/07	05/06	06/07
Experimental scenario 3	05/06	05/06	06/07

Table 6
Predicted effects of changes (explanations in each cell) in set-up and mating from 05/06 to 06/07 year on operating profit ($-values in each cell) for six farms (farm A–F).

Farm	Value of set-up change (NZ$/ha)	Value of mating change (NZ$/ha)	Value of set-up and mating change (NZ$/ha)
A	+6 Heifers calved earlier but cows were later	+162 PSM[a] 3 days later; treatment of periparturient disorders	+168 Net gain. Mainly due to lower non-pregnant rate and higher 6-week in-calf rate
B	+229 Better BCS. Tighter calving (heifers 1 week earlier)	−83 Mated 1 week less (resulting in 2% higher non-pregnant rate)	+146 Net gain. Mainly due to set-up. Cost (investment?) in shortening of mating period
C	−229 Lower Stocking Rate; Later PSC[b] but more compact calving	+15 Treatment of periparturient disorders; poorer bull EDE[c]	−214 Net loss. Mainly from lower stocking rate due to farm expansion
D	+63 Better BCS; 12% fewer 2–3 year cows	+152 Treatment of periparturient disorders; lower death rate; longer mating period	+215 Good gains overall, but more late-calving cows next year
E	−3 Higher stocking rate, but higher cow costs	+112 Treatment of periparturient disorders; more inclusive anestrous treatment; improved bull EDE	+109 Net gain mainly due to lower non-pregnant rate
F	−194 Slower calving dominated despite better cow condition	+230 Better AB[d] EDE due to heat mount detectors; better bull EDE due to bull management	+36 Small net gain

[a] Planned start of mating.
[b] Planned start of calving.
[c] Estrous detection efficiency.
[d] Artificial breeding.

A longer mating period in farm D contributed to good gains in 06/07, but is a retrograde strategy for improving reproductive performance in the longer term among seasonal pasture-based dairy farms.

Farm E has historically experienced unacceptably high non-pregnant rates. Modeling predicted a $109/ha improvement in profit through reducing non-pregnant rate, primarily by implementing strategies to improve EDE of bulls during the natural mating period.

In farm F the 4-week calving rate decreased from 80% to 70% resulting in a loss of NZ$194/ha despite an average increase of 0.5 in BCS. The improved EDE during both AB and bull mating in farm F resulted in a 2% increase in 6-week in-calf rate (71–73%) and a 4% decrease in non-pregnant rate (13–9%). This, together with a lower death rate at calving, resulted in a total value to farm profitability of approximately $230/ha.

4. Discussion

The reproductive modeling component is a recent addition to the WFM, and is constructed from well-known physiological requirements for postpartum cows to re-establish pregnancy. The default values and incidences that drive reproductive performance at the herd level within the WFM were derived from large-scale field observations. The utility of using the WFM to explore the on-farm consequences of altered reproductive performance or management policies represents a powerful tool for research and extension activities aimed at improving reproductive performance on pasture-based dairy farms.

General trends across all six farms showed that key set-up factors (farm conditions at the start of the farming year on 1 June) resulting in improved profitability were calving spread, stocking rate and BCS. The key factors in mating management were treatment of periparturient disorders, EDE during the AB period, and reproductive performance during natural bull mating. Although most farms gained substantially by improving these areas of mating management, the importance of farm set-up and how poor set-up conditions can negate gains made by subsequent mating management (e.g. farms C and F) was also indicated.

In 4 of the farms, EDE during bull mating had to be substantially reduced to <65% to get model predictions to fit the observed KPI in 05/06 year. This was surprising to most of the case study farmers, and was interpreted to mean that policies and activities of bull management during the bull mating were generally deficient. Farm F was an exception, showing the value of rotating and resting bulls properly and thereby improving EDE during bull mating from 60% to 90%. The difference in mating changes between farms C and E was largely attributed to improved bull EDE in farm E compared with farm C. The difference in gains was approximately $100/ha. The message about improved mating performance during the bull mating period was strong and recurrent. Farmers responded by reviewing their bull management policies in accordance with ‘best-practice’ industry recommendations (Burke et al., 2007); with particular attention to bull selection, managing 'working' bulls and ensuring sufficient numbers of bulls are with the herd during natural mating.

Modeling of the six farms highlighted that each farm has a unique set of conditions, which diminishes the value of applying general 'best-practice' advice for improving herd reproductive performance. For example, farm E is a hilly farm where cows walk long distances from the high lying plateau to the milking shed on the flats during peak lactation time. The energetic cost of this extraordinary walking activity is not accounted for by the WFM, and could have contributed to the unexplained differences between feed supplied and production variables (e.g. milksolids and end cow BW) for farm E.

Farms have to be analyzed individually and solutions are likely to be multi-facetted. Moreover, solutions will often require persistent implementation over several years before a measurable improvement in performance (Beukes et al., 2005). The interactions of set-up, mating and seasonal management in the farm system confounds the comparison of operating profit from year to year. This explains why farmers find it hard to measure financial benefits after changes in their management and why they often find it hard to justify these changes. This present exercise was not aimed at separating out the $-value of the individual mating management factors, but in a different study using 4 of these 6 farms, Beukes et al. (2007) showed the value of increasing EDE during AB, and therefore the 3-week submission rate from 80% to 90%, to be $80+/ha in the following year. Whole farm models are tools to analyze the farm system in a holistic way and can contribute to teasing out some of the complex interactions that occur over multiple years.

The present exercise also highlighted some areas for ongoing improvement to the WFM. One of these areas relates to the impact of BCS on PPAI. The consequences of PPAI being inaccurately predicted have flow-on effects in the WFM, with inaccurate rates of anestrus, anestrous cow treatments and conception rates in early mating. The default "BCS factor" in WFM is 8 days longer PPAI with each 1 unit decline in BCS at calving, which is an average value reported in several studies (McDougall et al., 1995; Burke et al., 2006; Burke and Roche, 2007). However, breed by age interactions on the magnitude of the "BCS factor" should also be taken into account. McDougall et al. (1995) found this "BCS factor" to be greater for Holstein-Friesian cows (15 days per unit BCS) than Jerseys (4 days per unit BCS). Two and three-year olds cows tend to have longer PPAI than mature cows. Burke et al. (1995) found that the interval to first estrus was increased from 62 to 85 days in Holstein-Friesian heifers calving at 1.5 BCS units (50 kg BW) lighter (4 versus 5.5 BCS). The "BCS factor" in this instance would amount to 15 days PPAI per unit BCS. The WFM can be manually adjusted to account for these interactions, but a high degree of user-expertise is required.

Another area highlighted for improvement was the magnitude with which changing BW during the 3 weeks preceding an insemination could influence conception rate. The intention was to account for the "rising plane of nutrition" effect on improved cow fertility, which although poorly defined, is generally believed to exist. Percent BW change is used instead of BCS since the early stages of positive energy balance are evident with increasing BW, without an obvious increase in BCS (Buckley et al., 2003; Roche et al., 2006). Internal reserves are replenished initially, before accumulation of peripheral adipose tissue that determines BCS. In an earlier version of the WFM, calculations for the BW factor (Eq. (2)) were derived from Irish data (Buckley et al., 2003). However, the work by Roche et al. (2006) using New Zealand data has shown that the parameters interpreted from Buckley et al. (2003) often result in the "rising plane of nutrition" effect having an overly large bearing on CR, both negatively during weight loss or positively during weight gain. Recent improvements in WFM allow the user access to the parameters controlling the "rising plane" effect and therefore control the magnitude of the effect of nutrition on CR. The default values now reflect the New Zealand data of Roche et al. (2006).

5. Conclusion

An empirical and deterministic representation of reproduction of individual cows in a whole farm model was a successful addition for modeling complex farm system interactions and exploring the impacts of key management factors on reproductive performance. Modeling six case study farms demonstrated that individual farms have unique characteristics that influence reproductive performance, but also commonly experienced challenges, such as poor performance during natural mating. The farmers accepted these findings and agreed there were management opportunities to improve reproductive performance in their dairy herd and operating profit of their dairy business.

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Systemic antibiotic treatment of clinical endometritis in dairy cows with ceftiofur or two doses of cloprostenol in a 14-d interval

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ABSTRACT

The objective of the study was to compare the systemic antibiotic treatment of clinical endometritis in dairy cows with ceftiofur with a treatment protocol consisting of two doses of prostaglandin $F_{2\alpha}$ analogue cloprostenol in a 14-d interval. On 2 commercial dairy farms, housing a total of 1900 Holstein cows, all cows that calved between June 2008 and January 2009 were examined 21–27 d in milk (DIM) by vaginoscopy. Cows with clinical signs of endometritis, i.e. vaginal discharge containing flecks of pus, mucopurulent material or purulent mucus, were randomly allocated to one of two treatment groups. Cows in group CEF ($n = 141$) received 1 mg/kg BW of ceftiofur (i.m.) on 3 consecutive days. Cows in group CLP ($n = 140$) received 0.5 mg of cloprostenol (i.m.) at the day of enrolment and 14 d later. All cows were re-examined by vaginoscopy 42–48 DIM. Proportion of cows cured, i.e. cows with clear, translucent or no mucus, 42–48 DIM (74.2 and 80.2% in groups CEF and CLP, respectively) was not affected by treatment group ($P = 0.09$). The voluntary waiting period was set at 40 DIM. Artificial insemination (AI) submission rate, days to first service, first service conception rate, days open and proportion of cows pregnant did not differ between the groups. In conclusion, the systemic treatment with 1.0 mg/kg BW of ceftiofur on 3 consecutive days in cows with signs of clinical endometritis 21–27 DIM was equivalent to an intervention protocol consisting of two doses of cloprostenol in a 14-d interval.

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1. Introduction

Endometritis is one of the most common disorders in dairy cows, causing decreased fertility and high economic losses. Recently, a consortium of authors defined clinical endometritis as the presence of purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) uterine exudate in the vagina, 21 d or more postpartum, and not accompanied by systemic signs (Sheldon et al., 2006a). A scoring scheme for vaginal discharge ranging from clear or translucent mucus (clinically healthy) to purulent discharge has been suggested by Williams et al. (2005). The relevance of mild endometritis, defined by mucus containing flecks of pus, and its effect on reproductive performance is contradictory (Dohmen et al., 1995; LeBlanc et al., 2002a; Pleticha et al., 2009). Although it has been demonstrated that mild clinical endometritis does not effect fertility (LeBlanc et al., 2002a), studies on subclinical endometritis found that in cows with translucent, clear or no mucus but elevated number of polymorphonuclear neutrophils (PMN) in endometrial samples, reproductive performance is negatively affected (Kasimanickam et al., 2004; Kaufmann et al., 2009; Sheldon et al., 2009).

The most prevalent pathogens involved in the aetiology of endometritis are *Arcanobacterium pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum*, and *Prevotella* sp. (Sheldon et al., 2002; Földi et al., 2006; Sheldon et al., 2006a). Suggested treatment protocols for clinical endometritis include the intrauterine administration of

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antibiotics (LeBlanc et al., 2002b; Drillich et al., 2005a; Runciman et al., 2008) or the administration of $PGF_{2\alpha}$ and its analogues (Heuwieser et al., 2000; LeBlanc et al., 2002b; Drillich et al., 2005a; Drillich et al., 2005b). The intrauterine infusion of antiseptics, e.g. Lugol's solution, has been tested, but some authors found negative effects on future fertility of the cows (Heuwieser et al., 2000; Knutti et al., 2000). Furthermore, treatment approaches with proteolytic enzymes (Drillich et al., 2005a), the administration of estradiol (Pepper and Dobson, 1987; Sheldon and Noakes, 1998) or GnRH (Janowski et al., 2001) have been described.

A systemic antibiotic treatment of cows with clinical endometritis, however, as described for acute metritis (Drillich et al., 2001; Zhou et al., 2001; Chenault et al., 2004), has not been reported in the recent literature. In an older study, Masera et al. (1980) found in cows with chronic endometritis higher concentrations of oxytetracycline in plasma after intramuscular administration than after intrauterine administration. For both routes, oxytetracycline was detectable in endometrial tissue. Pharmacokinetic data for a systemic administration of 1 mg/kg BW ceftiofur suggest an efficacious concentration of the antibiotic in the endometrium in early postpartum cows, i.e. within 3 dpp, up to 24 h after treatment (Okker et al., 2002; Drillich et al., 2006). In Europe, ceftiofur is approved for the treatment of acute metritis during the first 10 dpp at a dosage of 1 mg/kg BW. Pharmacokinetic data on intrauterine applied antibiotics have rarely been published in the recent literature (Braun et al., 2009). Bretzlaff et al. (1983) and Masera et al. (1980) described that the intrauterine administration of oxytetracycline achieves sufficient concentration in the endometrium, but little penetration to deeper layers of the uterus. In contrast to systemically applied antibiotics, it has been assumed that the presence of pus and organic debris in the uterine fluids decreases the efficacy of intrauterine antibiotics (Paisley et al., 1986). Clinical studies, however, demonstrated the efficacy of an intrauterine infusion of antibiotics in cows with clinical endometritis (LeBlanc et al., 2002b; Runciman et al., 2008).

The hypothesis tested in the study was that in cows with clinical endometritis 21–27 dpp a systemic treatment with 1 mg/kg BW of ceftiofur on 3 consecutive days results in improved clinical cure and conception rates compared with a protocol consisting of two treatments with the $PGF_{2\alpha}$ analogue cloprostenol in a 14-d interval.

2. Materials and methods

2.1. Study animals

The study was conducted between June 2008 and January 2009 on two commercial dairy farms in Brandenburg, Germany. Herd sizes were 851 (herd A) and 1068 (herd B) cows, respectively. Animals were housed in free-stall facilities with cubicles, rubber mats and slatted floors all year round. Calving pens were straw-bedded. On both farms, cows were fed a total mixed ration based on maize silage, grass silage and concentrates. Herd average milk yields were 9200 kg (fat 4.2%, protein 3.3%) and 9300 kg per lactation (fat 4.2%, protein 3.3%) in herds A and B, respectively.

2.2. Study design

The participating farmers and the local veterinarian were informed about all relevant characteristics of the study and agreed with the study design (informed consent). The veterinarians and AI technicians involved in the study, however, were not informed about the allocation of cows to the treatment groups. All cows were examined by vaginal inspection 21–27 dpp. The vulva was cleaned with a dry paper towel before a speculum (Hauptner and Herberholz, Solingen, Germany) was inserted into the vagina. Each cow was examined with a speculum that had been autoclaved and single packed again at the Clinic for Animal Reproduction, Berlin. Specula were used only once on the farm and autoclaved again before the next use. Each speculum was unpacked and moistened with 0.9% saline immediately before examination. The speculum was inserted into the vagina up to the exterior cervical os; illumination was provided by an electric torch. Classification of uterine health (healthy; mild, moderate, severe endometritis) was made according to a vaginal discharge score (VDS) suggested by Williams et al. (2005). Clear or translucent mucus is equivalent to score 0 for healthy cows. Score 1 describes mucus containing flecks of white or off-white pus. Score 2 describes discharge containing less than 50% white or off-white mucopurulent material. In score 3 the discharge is composed of more than 50% white or yellow pus or is sanguineous. To avoid an effect of an antibiotic treatment not related to the objective of the study, cows that received a systemic antibiotic treatment prior to 21 DIM, e.g. for acute metritis were excluded from the study. Additionally, cows with vaginal lesions or a caesarean section and cows that were not supposed to be bred after calving were excluded from the study. Cows that received a systemic antibiotic treatment between 21 DIM and the re-examination 42–48 DIM for purposes not related to the study, e.g. acute mastitis, were retrospectively withdrawn.

Cows diagnosed with $VDS > 0$ at 21–27 DIM and not meeting the exclusion criteria were enrolled in the study. Animals were systematically allocated to one of two groups according to their ear tag numbers. In group CEF (even ear tag numbers), cows received a treatment with 1 mg/kg of estimated BW of ceftiofur (Excenel RTU, Pfizer Tiergesundheit, Berlin, Germany, i.m.) on 3 consecutive days, beginning at the day of enrollment. In the U.S. and in Europe, ceftiofur is approved for acute postpartum metritis. Administration of ceftiofur to cows with clinical endometritis would be considered an extra label use. Thus, for the study an experimental test permit was obtained from the responsible authorities.

In group CLP (odd ear tag numbers), cows received 0.5 mg of cloprostenol (PGF Veyx forte, Veyx Pharma, Schwarzenborn, Germany, i.m.) at the day of enrollment and were re-treated 14 d later. All cows enrolled in groups CEF and CLP were re-examined 42–48 DIM by vaginal examination as described above, except cows that had already been bred by AI.

On both farms the voluntary waiting period (VWP) was set at 40 DIM. Estrus detection was performed by the farm staff twice a day. Cows found in estrus were examined by an AI technician by vaginoscopy and rectal palpation and bred by AI, if confirmed in estrus and VDS 0. Animals not inseminated by 70 DIM were examined by the local veterinarians by vaginoscopy and rectal palpation for abnormalities of the genital tract and treated according to their findings. Cows with a corpus luteum received 0.5 mg of cloprostenol to induce estrus whereas cows with cystic ovaries (fluid-filled structure >2.5 cm in diameter, cow not seen in estrus) received 0.05 mg of GnRH analogue lecirelin (Dalmarelin, Selectavet, Weyarn-Holzolling, Germany, i.m.). Cows without a corpus luteum detectable and without pathological findings of the ovaries and uterus received an Ovsynch protocol (Pursley et al., 1997). These examinations were repeated in a 14-d interval. Pregnancy diagnosis was performed between 42 and 49 d after AI by transrectal palpation of the uterus and its contents by the same veterinarian on both farms.

2.3. Reproductive parameters

Clinical cure rate was defined as the proportion of cows with no signs of clinical endometritis, i.e. VDS 0, at the re-examination 42–48 DIM. Cows that had already been inseminated before the re-examination were regarded as cured. Reproductive performance was characterized by days from calving to first AI, 21 d submission rate (number of cows inseminated for the first time within 21 d after VWP divided by number of all cows after VWP), first service conception rate (FSCR) (number of cows pregnant after first AI divided by number of cows inseminated × 100), days to pregnancy, proportion of cows pregnant by 200 DIM (number of cows pregnant by 200 DIM divided by number of cows enrolled × 100). Cows not pregnant by 200 DIM were regarded as open, even if they remained in the herd and conceived later in lactation. Cows inseminated but culled before pregnancy diagnoses were regarded as not pregnant. All diagnoses and treatments were documented on case report forms. At the end of the study, case report forms were checked for compliance.

2.4. Statistical analyses

Data were analyzed using SPSS (Version 16.0, SPSS Inc., Munich, Germany). The prevalence of endometritis was compared between herds A and B and between primiparous and multiparous cows by χ^2-analysis. Binary logistic regression models were calculated for the risk of clinical cure at the re-examination and for the risk of conception at first AI as outcome variables. Survival analyses for the hazards of insemination and pregnancy within 200 DIM, respectively, were performed using Cox-regression, censoring cows that were not inseminated and not pregnant, respectively. For logistic regression and Cox-regression, cows culled before pregnancy diagnoses were regarded as inseminated but not pregnant. For logistic regression models as well as for Cox-regression, VDS 21–27 DIM (1 = mucus with flecks, 2 = <50% pus, 3 = >50% pus), group (0 = CLP, 1 = CEF), herd (0 = herd A, 1 = herd B), and parity class (0 = primiparous, 1 = multiparous) were included as factors. In addition, for logistic regression model induction of estrus at first AI with $PGF_{2\alpha}$ or Ovsynch (0 = no induction of estrus, 1 = induced estrus) was included as factor. Interactions of treatment group × herd have been included in the analyses.

Adjusted odds ratios, hazard ratios, confidence intervals (CI), and *P*-values are reported. For logistic regression as well as for survival analyses, CI was set at 95%. For all statistical analyses level of significance was set at $\alpha = 0.05$.

3. Results

A total of 1164 Holstein-Friesian cows that had calved between June and November 2008 were examined by vaginoscopy at 21–27 DIM (657 cows in herd A, 507 cows in herd B). The prevalence of endometritis 21–27 DIM was 39.7 and 42.2% in herds A and B, respectively, and did not differ between the herds ($P = 0.39$).

After checking the exclusion criteria, 193 cows were withdrawn from further analyses because of receiving a systemic antibiotic treatment for e.g. acute metritis or meeting other exclusion criteria. One cow with incomplete treatments (group CLP) was retrospectively withdrawn from the study. Finally, a total of 281 cows with endometritis score 1–3 were eligible for the final analyses (herd A: 70 in CEF, 75 in CLP; herd B: 71 in CEF, 65 in CLP). Proportion of primiparous cows was 41.8 and 38.6% in CEF and CLP, respectively ($P = 0.58$).

The overall proportion of cows cured 42–48 DIM was 74.2 and 80.2% in CEF and CLP, respectively (herd A: 68.7% in CEF, 77.6% in CLP; herd B; 80.0% in CEF, 83.3% in CLP). The proportion of cows diagnosed with VDS 1–3 at the re-examination did not differ between the 2 groups (Table 1). A total of 28 cows were not re-examined because of culling (2 in CEF, 5 in CLP) or logistic reasons (7 in CEF, 14 in CLP). The clinical cure at the re-examination was affected by vaginal discharge score, with a lower likelihood for clinical cure for cows with VDS 3 compared with VDS 1 at first examination (Table 2).

First service conception rate was greater (32.1% vs 20.9%) in CLP than in CEF. No differences between treatments were found in 21 d submission rate, days to first service, proportion of cows bred by 200 DIM, days to pregnancy and the cumulative proportions of cows pregnant or culled within the observation period (Table 3). Estrus was induced at first AI (by the use of $PGF_{2\alpha}$ or Ovsynch) in 42 and 37 cows in CEF and CLP, respectively.

Further analysis by logistic regression revealed no effect of treatment group on FSCR, but effects of herd ($P = 0.05$) and interactions of treatment group by herd ($P = 0.02$). No effects were found for VDS assigned 21–27 DIM, induction of estrus at first AI or parity class (Table 4).

Cox-regression analyses found no effect of treatment group on the likelihood of insemination within 200 DIM, however, an effect of VDS and parity class was found. The chance for insemination was lower for cows with VDS 2 or 3 compared with score 1 (HR = 0.74, $P = 0.04$, and HR = 0.67, $P = 0.01$, respectively) and lower for multiparous cows compared with primiparous cows (HR = 0.58, $P < 0.01$). The chance for pregnancy within 200 DIM was lower for multiparous cows compared with primiparous cows (HR = 0.63, $P < 0.01$). No effect was found for treatment group, VDS, and herd (Table 5).

Table 1
Classification of vaginal discharge score 21–27 DIM and 42–48 DIM in cows treated with 1.0 mg/kg BW of ceftiofur on 3 consecutive days or 0.5 mg of cloprostenol at 21–27 and 35–41 DIM.

Treatment group[a]	Examination DIM	*n*	Vaginal discharge score (%)[b]			
			Score 0	Score 1	Score 2	Score 3
CEF	21–27	141	–	44.7	31.2	24.1
	42–48	133	74.2	14.4	3.0	8.3
CLP	21–27	140	–	36.4	32.1	31.4
	42–48	120	80.2	14.0	3.3	2.5

[a] CEF, cows with endometritis 21–27 DIM received 1.0 mg/kg BW of ceftiofur on 3 consecutive days; CLP, cows with endometritis 21–27 DIM received 0.5 mg of cloprostenol 21–27 and 35–41 DIM.

[b] Vaginal discharge score: 0 = clear or translucent mucus at vaginal examination, no signs of endometritis; 1 = mucus with flecks of white or off-white pus; 2 = discharge with less than 50% white or off-white mucopurulent material; 3 = discharge with more than 50% white or yellow pus.

Table 2
Results of binary logistic regression analysis for the risk of clinical cure at a re-examination in cows with clinical endometritis 21–27 DIM treated with 1.0 mg/kg BW of ceftiofur on 3 consecutive days or 0.5 mg of cloprostenol at 21–27 and 35–41 DIM.

Factor	Clinical cure at re-examination		
	OR[a]	CI 95%[b]	*P*
Treatment group[c]	0.49	0.22–1.13	0.09
Vaginal discharge score[d]			<0.01
Score 1		Reference score	
Score 2	1.15	0.50–2.65	0.74
Score 3	0.24	0.11–0.49	<0.01
Parity class[e]	0.68	0.35–1.32	0.25
Herd[f]	1.17	0.14–9.57	0.88
Treatment group × herd	1.45	0.40–5.24	0.57
Constant	14.87		<0.01

[a] OR, adjusted odds ratio.

[b] CI, confidence interval.

[c] Treatment group: 0 = group CLP, cows with endometritis 21–27 DIM received 0.5 mg of cloprostenol 21–27 and 35–41 DIM; 1 = group CEF, cows with endometritis 21–27 DIM received 1.0 mg/kg BW of ceftiofur on 3 consecutive days.

[d] Vaginal discharge score at 21–27 DIM: 1 = mucus containing flecks of white or off-white pus, 2 = discharge containing less than 50% white or off-white mucopurulent material, 3 = discharge is composed of more than 50% white or yellow pus.

[e] Parity class: 0 = primiparous, 1 = multiparous.

[f] Herd: 0 = herd A, 1 = herd B.

Table 3
Descriptive reproductive performance traits in cows with clinical endometritis 21–27 DIM treated with 1.0 mg/kg BW of ceftiofur on 3 consecutive days or 0.5 mg of cloprostenol at 21–27 and 35–41 DIM.

Outcome	Treatment group[a]	
	CEF	CLP
21 d submission rate, %	36.8 (50/136)	29.3 (39/133)
(*n*/*n*)	28.3–44.5	21.2–36.7
95% CI[b]		
Median days to first AI	69.0	69.0
IR[c]	51.8–79.8	54.0–85.0
Cows inseminated at	95.0 (134/141)	93.6 (131/140)
200 DIM, % (*n*/*n*)	91.1–98.3	89.2–97.3
95% CI		
First AI conception	20.9 (28/134)	32.1 (42/131)
rate, % (*n*/*n*)	13.6–27.4	23.7–39.7
95% CI		
No. of inseminations	378	350
Proportion pregnant at	65.2 (92/141)	64.3 (90/140)
200 DIM, % (*n*/*n*)	57.0–75.8	56.0–71.9
95% CI		
Inseminations per pregnancy	4.4	3.9
Median days open	101	94
IR	79.0–128.8	65.0–121.8

[a] CEF, cows with endometritis 21–27 DIM received 1.0 mg/kg BW of ceftiofur on 3 consecutive days; CLP, cows with endometritis 21–27 DIM received 0.5 mg of cloprostenol 21–27 and 35–41 DIM.

[b] CI, confidence interval.

[c] IR, interquartile range.

Table 4
Results of binary logistic regression analysis for the risk of conception after first AI in cows with clinical endometritis 21–27 DIM treated with 1.0 mg/kg BW of ceftiofur on 3 consecutive days or 0.5 mg of cloprostenol at 21–27 and 35–41 DIM.

Factor	Conceiving after first AI		
	OR[a]	CI 95%[b]	*P*
Treatment group[c]	0.93	0.44–1.97	0.86
Vaginal discharge score[d]			0.86
Score 1		Reference score	
Score 2	1.20	0.61–2.37	0.59
Score 3	1.04	0.50–2.17	0.91
Induced estrus[e]	0.54	0.27–1.07	0.08
Parity class[f]	0.75	0.41–1.40	0.34
Herd[g]	6.24	1.03–37.90	0.05
Treatment group × herd	0.23	0.07–0.77	0.02
Constant	0.57		0.38

[a] OR, adjusted odds ratio.

[b] CI, confidence interval.

[c] Treatment group: 0 = group CLP, cows with endometritis 21–27 DIM received 0.5 mg of cloprostenol 21–27 and 35–41 DIM; 1 = group CEF, cows with endometritis 21–27 DIM received 1.0 mg/kg BW of ceftiofur on 3 consecutive days.

[d] Vaginal discharge score: 1 = mucus containing flecks of white or off-white pus, 2 = discharge containing less than 50% white or off-white mucopurulent material, 3 = discharge is composed of more than 50% white or yellow pus.

[e] Induced estrus: 0 = no induction of estrus, 1 = estrus induced by administration of $PGF_{2\alpha}$ or Ovsynch.

[f] Parity class: 0 = primiparous, 1 = multiparous.

[g] Herd: 0 = herd A, 1 = herd B.

4. Discussion

The treatment of clinical endometritis has been the objective of numerous studies in recent years (e.g. Heuwieser et al., 2000; Knutti et al., 2000; LeBlanc et al., 2002b; Drillich et al., 2005a; Drillich et al., 2005b; Runciman et al., 2008). The administration of $PGF_{2\alpha}$ and its analogues has been described as an adequate intervention (Heuwieser et al., 2000; LeBlanc et al., 2002b; Drillich et al., 2005a; Drillich et al., 2005b). Even though $PGF_{2\alpha}$ is widely used as an intervention strategy for endometritis, science-based evidence of its effect on cows with endometritis but without corpus luteum has to be demonstrated, yet. For this reason we administered two doses of cloprostenol in a 14-d interval to ensure that in all cyclic cows at least once a corpus luteum was present (Heuwieser et al., 2000). Studies on the intrauterine administration of antibiotics showed that this strategy was effective (Runciman et al., 2009) and superior than or equal to the use of prostaglandins (LeBlanc et al., 2002b; Drillich et al., 2005b). The treatment of clinical endometritis with a systemic administration of antibiotics has not been published in recent literature.

In up to 88% of postpartum cows, i.e. up to 28 dpp, bacteria were isolated from the genital tract and in 64% of cows uterine pathogens, e.g. *E. coli*, have been diagnosed (Williams et al., 2005). In uterine samples of cows with endometritis 60.3% were positive for bacteria, whereas only 41.8% of the healthy samples showed bacterial growth (Petit et al., 2009). Furthermore, the density of pathogen growth was higher in cows with endometritis than in healthy cows. Therefore, an antibiotic treatment of endometritis seems to be rational. The hypothesis tested in

Table 5
Results of the survival analyses (Cox-regression) for the hazard of insemination and pregnancy in cows with clinical endometritis 21–27 DIM treated with 1.0 mg/kg BW of ceftiofur on 3 consecutive days or 0.5 mg of cloprostenol at 21–27 and 35–41 DIM.

Factor	Insemination			Pregnancy		
	HR[a]	CI 95%[b]	*P*	HR	CI 95%	*P*
Treatment group[c]	1.04	0.75–1.46	0.80	1.08	0.72–1.62	0.72
Vaginal discharge score[d]			0.02			0.95
Score 1		Reference score			Reference score	
Score 2	0.74	0.55–0.99	0.04	0.98	0.69–1.39	0.92
Score 3	0.67	0.49–0.90	0.01	0.95	0.66–1.36	0.76
Parity class[e]	0.58	0.45–0.75	<0.01	0.63	0.46–0.85	<0.01
Herd[f]	0.77	0.36–1.66	0.50	1.56	0.61–3.93	0.35
Treatment group × herd	1.01	0.62–1.64	0.98	0.76	0.43–1.37	0.36

[a] HR, adjusted hazard ratio.

[b] CI, confidence interval.

[c] Treatment group: 0 = group CLP, cows with endometritis 21–27 DIM received 0.5 mg of cloprostenol 21–27 and 35–41 DIM; 1 = group CEF, cows with endometritis 21–27 DIM received 1.0 mg/kg BW of ceftiofur on 3 consecutive days.

[d] Vaginal discharge score: 1 = mucus containing flecks of white or off-white pus, 2 = discharge containing less than 50% white or off-white mucopurulent material, 3 = discharge is composed of more than 50% white or yellow pus.

[e] Parity class: 0 = primiparous, 1 = multiparous.

[f] Herd: 0 = herd A, 1 = herd B.

the present study was that a systemic antibiotic treatment with ceftiofur would result in improved clinical cure and conception rates followed by a greater proportion of cows pregnant at 200 DIM compared with a group treated with cloprostenol. This hypothesis, however, has to be rejected for cows included in this study. It should be emphasized that cows with a history of acute metritis and treated with antibiotics prior to the diagnosis of clinical endometritis were withdrawn from the study. Proportion of cows cured did not differ between the 2 groups; reproductive performance traits were not affected by treatment. Considering the numerical differences and the statistical power of the study it cannot be completely excluded that this may have been caused by a type II error.

Pathogens predominantly associated with endometritis, i.e. *E. coli* and *A. pyogenes* (Dohmen et al., 1995; Williams et al., 2005) should be susceptible to ceftiofur (Sheldon et al., 2004). The systemic administration of ceftiofur in early postpartum cows results in concentrations that exceed the minimum inhibitory concentrations for pathogens associated with uterine disorders (Okker et al., 2002; Sheldon et al., 2004; Drillich et al., 2006) and has been described as effective in the treatment of metritis (Drillich et al., 2001; Chenault et al., 2004; Sheldon et al., 2004). Information on the pharmacokinetics of ceftiofur in cows with clinical endometritis ≥21 DIM, however, is not available. It can be hypothesized that with the present study design concentrations of ceftiofur did not exceed the minimum inhibitory concentrations required to eliminate uterine pathogens. Further research is required to evaluate whether using a larger dose of 2.2 mg of ceftiofur per kg of BW, which is approved in the U.S., or a longer treatment period would result in improved cure rates or reproductive performance traits.

The knowledge of immunological response of endometrial cells to uterine pathogens has dramatically increased in the last years, as reviewed by Sheldon et al. (2009). Recent research suggests that the use of $PGF_{2\alpha}$ for the treatment of endometritis is efficient not only by induction of myometrial contractions and estrus (Weems et al., 2006), but also by regulation of inflammatory responses in the endometrium. It has been demonstrated that bacterial endotoxin lipopolysaccharide, expressed by uterine pathogens such as *E. coli* switch the endometrial epithelial secretion of prostaglandins from the F to the E series. This results in prolonged luteal phases and modulation of endocrine functions that are essential for reproduction (Herath et al., 2009; Sheldon et al., 2009). Therefore, the exogenous administration of $PGF_{2\alpha}$ might compensate the reduced endogenous epithelial secretion of $PGF_{2\alpha}$. In contrast, other authors reported that lipopolysaccharide stimulates the secretion of $PGF_{2\alpha}$ (Leung et al., 2001). The link between the described findings in the early postpartum period and the pathogenesis of clinical endometritis is still not fully understood. Results by Gabler et al. (2009) suggested an association between a dys-regulated cytokine and/or prostaglandin profile in the endometrium and the occurrence of clinical and subclinical endometritis. Furthermore, there is an ongoing discussion in human and veterinary medicine about the protective effects of microbial biofilms produced, e.g. by *E. coli* and *Staphylococcus* spp. that decrease the efficacy of antimicrobials (Stewart and Costerton, 2001; LeBlanc and Causey, 2009).

It is widely accepted that clinical endometritis has a negative impact on reproductive performance (LeBlanc et al., 2002a; Sheldon et al., 2006b; Runciman et al., 2008). Furthermore, it has been demonstrated that the proportion of cows pregnant by 200 DIM decreased with an increasing VDS (Falkenberg and Heuwieser, 2005; Pleticha et al., 2009). The present study partly confirmed findings that a VDS 2 and 3 (mucopurulent and purulent vaginal discharge) has a negative impact on fertility (McDougall et al., 2007; Pleticha et al., 2009). We found that the chance for insemination was lower in cows with VDS 2 and 3 compared with VDS 1. No effect, however, was found for VDS 2 and 3 compared to VDS 1 on FSCR and likelihood of pregnancy. It remains unclear, if this should be interpreted as a positive effect of both treatment protocols or an effect of delayed insemination in groups VDS 2 and 3. It is questionable if cows with VDS 1 need to be treated. LeBlanc et al. (2002b) found that mild clinical endometritis (VDS 1) has no negative impact on fertility. This, however, is contradictory to findings that even cows categorized as VDS 0 but with increased proportion of PMN, i.e. subclinical endometritis, showed a reduced fertility (Kasimanickam et al., 2004).

Most authors agree that vaginal examination with a speculum is accurate for the diagnosis of endometritis (LeBlanc et al., 2002a; Sheldon et al., 2006a; Runciman et al., 2008), although the absence of discharge in the vagina does not always reflect the absence of inflammation in the uterus (Kasimanickam et al., 2004; Sheldon et al., 2006a; Barlund et al., 2008). Result of vaginal examination has a higher predictive value for time to pregnancy than rectal palpation (LeBlanc et al., 2002a). In this study, clinical endometritis was diagnosed by vaginal examination with a speculum. Vaginal examination can also be performed by using a gloved hand or the Metricheck device (Sheldon et al., 2002; McDougall et al., 2007; Pleticha et al., 2009). Finally, it remains unclear, if clinical cure rates and reproductive performance traits resulted from the administration of ceftiofur, cloprostenol or from self-recovery, i.e. both treatment strategies were ineffective. Because of the owners concerns on economical losses in a group with no intervention strategy, it was not possible to include an untreated control group. Only few studies have been published that included an untreated control group (LeBlanc et al., 2002b; Runciman et al., 2009). Recently, Runciman et al. (2009) found that cows diagnosed with endometritis 7–28 DIM benefit from an intrauterine infusion of 500 mg of cephapirin. Treated cows showed an improved reproductive performance compared with untreated controls.

It can be assumed that management practices on the farms had an effect on fertility traits. Cows were bred after a relatively short VWP of 40 DIM. Therefore, it could be hypothesized that a longer VWP with an extended interval for reconstitution of the endometrium would have resulted in higher conception rates in one of the groups. This hypothesis is partly supported by the fact that time to insemination was affected by VDS, but FSCR and proportion of cows pregnant were not. Submission rate and days to first service, however, were similar in both groups.

Therefore, VWP might have influenced the overall conception rates but did not affect the results of the comparison between the groups. The management practice to use $PGF_{2\alpha}$ or an Ovsynch protocol to induce estrus and ovulation in cows not bred by 70 DIM did not affect FSCR. The design of this study, however, does not allow conclusions about the efficacy of these management tools.

Some studies found that parity is another factor that affects conception rates (Cartmill et al., 2001; Tenhagen et al., 2004; Kaufmann et al., 2009) or the chance of insemination and pregnancy (Drillich et al., 2006; Pleticha et al., 2009) while others did not (Lincke et al., 2006). Therefore, this trait was included in the logistic regression model and in Cox-regressions. In the present study, multiparous cows had a lower likelihood of insemination and pregnancy, respectively, than primiparous cows. No effect, however, was found for parity on FSCR.

For veterinarians in dairy practice, treatment decision includes also considerations about a prudent use of drugs and the cost per treatment. In addition, veterinarians should be aware of a growing public concern about a possible association between the use of antibiotics in food producing animals and multi-drug resistances of bacteria against antibiotics in human medicine.

5. Conclusion

The present study demonstrated that the systemic treatment with 1.0 mg/kg BW of ceftiofur on 3 consecutive days in cows with signs of clinical endometritis 21–27 DIM was not superior to a treatment protocol consisting of two doses of cloprostenol in a 14-d interval. In two herds with a VWP of 40 dpp, reproductive performance did not differ between the two treatment protocols. Further research should focus on the mechanisms of action of prostaglandin $F_{2\alpha}$ in cows with endometritis on future fertility. More information on the pharmacokinetics of ceftiofur in cows with clinical endometritis is required to determine reasons for treatment failures in the present study and to provide more detailed information for future treatment opportunities, e.g. with different regimes of an antibiotic therapy.

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The effect of body condition at calving and supplementation with *Saccharomyces cerevisiae* on energy status and some reproductive parameters in early lactation dairy cows

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ABSTRACT

Improving the energy status of dairy cows during the early post-partum (PP) period by adding a safe dietary supplement such as live yeast culture (YS) may have a positive effect on reproductive function. The objective was to examine potential benefits of YS supplementation on PP energy status and fertility indices of dairy cows managed to have low or high body condition score (BCS, 1–5 scale) at calving. Forty (10 primiparous and 30 multiparous) Holstein/Friesian dairy cows were blocked by yield, parity, BCS, and predicted calving date. Within each block, cows were randomly allocated to a 2 × 2 factorial arrangement of treatments which were: BCS at calving (low $\leq$3.5 or high $\geq$3.75; $n = 20$) and YS supplementation (2.5 g/cow/day for pre-calving and 10 g/cow/day for post-calving $\times 10^8$ CFU of *Saccharomyces cerevisiae*/g) (supplemented or control; $n = 20$). Daily milk yield was recorded and weekly milk composition, BCS and BW were assessed from calving to week 10 PP. Forage (100% grass silage pre-calving; 50% grass silage, 50% maize silage post-calving; *ad libitum*) intake was recorded individually. Concentrate (2 kg of pre-calver nuts ± YS for pre-calving and 8 kg of lactating nuts ± YS for post-calving) feeding was controlled individually. Estimated energy balance PP was calculated on a weekly basis individually as the difference between the net energy (NE) intake and the sum of NE for maintenance and milk production. Insulin and IGF-I concentrations were determined on days 14 and 7 pre-calving and 1, 5, 15, 25 and 35 post-calving. Daily ovarian ultrasonography was performed from day 10 PP to monitor the size and development of the first dominant follicle (>10 mm in diameter with absence of other large growing follicles), first ovulatory follicle and days to first ovulation PP. Pre-ovulatory peak of serum oestradiol concentration was determined during the 2 days before ovulation day. Cows with high BCS (over-conditioned) at calving ingested less NE, produced more milk NE output, and consequently had a significantly ($P < 0.05$) exacerbated negative energy balance in comparison with low BCS cows (moderately conditioned) during early lactation. Higher ($P < 0.05$) insulin concentrations and a tendency for higher ($P = 0.06$) pre-ovulatory peak oestradiol concentrations in low BCS group were detected in the early PP period. Supplementing the diet with YS had no effect ($P > 0.10$) on NE intake, NE milk output or energy balance. On the other hand it increased ($P < 0.01$) insulin concentration and tended to increase ($P = 0.07$) pre-ovulatory peak oestradiol concentrations and the size of first ovulatory follicle ($P = 0.09$) early PP. Feeding YS had no effect on energy status of lactating dairy cows with high or low BCS at calving, whilst it improved serum insulin concentration, pre-ovulatory peak of oestradiol and the size of first ovulatory follicle in the early PP period. These observed effects of YS supplementation require to be substantiated with further research.

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1. Introduction

High genetic merit dairy cows in early lactation do not consume sufficient feed to meet their energy requirements for maintenance and milk secretion. To cope with this shortfall in nutrient balance, fat and protein stored in body reserves are mobilized and used for required physiological functions. Consequently, most dairy cows enter into a period of negative energy balance (NEB) during the early post-partum (PP) period. Body condition score (BCS) at calving has been shown to have a marked effect on energy balance in early lactation (Garnsworthy and Topps, 1982; Wathes et al., 2007a). Furthermore, NEB during early lactation has a well documented deleterious effect on dairy cow reproductive performance (Beam and Butler, 1999; Butler, 2003; Wathes et al., 2007b). Thus, altering BCS at calving provides an opportunity to assess the effect of dietary supplements on NEB of differing severities within the same experiment.

Feed intake and efficiency of digestion are important determinants of energy status in early lactation and thus it is appropriate to investigate the effect of supplements that may improve energy balance and reproductive performance. One of these dietary supplements is the live yeast culture (YS) of the strain *Saccharomyces cerevisiae*^{1026} which has been widely used to alter rumen fermentation and enhance ruminal digestive function. Most of the work with this supplement has been performed on fistulated dry cows or steers or even *in vitro*. On the other hand, results of the limited research conducted with dietary yeast supplement in dairy cows during the transition period have been inconsistent. Various studies have reported beneficial effects of YS supplementation on DMI, ration digestibility, and milk yield and composition (Wohlt et al., 1998; Robinson and Garrett, 1999; Dann et al., 2000). In contrast, others have found no significant benefits of YS supplementation (Robinson, 1997; Soder and Holden, 1999). Bruno et al. (2009a) reported no effect of YS on reproduction of multiparous cows under heat stress. However, no studies have investigated the effect of YS supplementation on reproductive performance in cows not under heat stress in component fed herds. Furthermore, no studies have determined if the effect of YS supplementation on dairy cow reproductive performance differs between cows with high BCS at calving (expected to have severe NEB) and cows with moderate BCS at calving (expected to have moderate NEB).

Therefore, the objectives of this experiment were, to investigate the effect of supplementing YS (*S. cerevisiae*^{1026}) to the diet of Holstein/Friesian dairy cows beginning at approximately 14 days pre-calving until day 70 of lactation on net energy (NE) intake, milk NE output, energy balance (EB) and some reproductive indices in dairy cows that have high or low BCS at calving.

2. Materials and methods

2.1. Animals and experimental design

All procedures involving animals were approved by the Animal Research Ethics Committee (University College Dublin) and conducted under experimental license from the Irish Department of Health and Children in accordance with the Cruelty to Animals Act 1876, and the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994, for the use of live animals in experiments. Forty (10 primiparous and 30 multiparous) Holstein/Friesian dairy cows were housed in free stall facilities equipped with a computerized individual feed intake recording system (Insentec B.V. Repelweg 10, 8316 PV Marknesse, Holland) and with *ad libitum* access to fresh water 21 days before their expected calving date until 70 days in milk (DIM). Cows were blocked by previous lactation 305-d milk yield, parity, BCS, and predicted calving date. Within each block, cows were randomly allocated to a 2 × 2 factorial arrangement of treatments approximately 60 days before parturition.

2.2. Treatments and feeding management

Treatments were imposed from day 14 pre-calving until day 70 of lactation. The main effects in this factorial experiment were: (1) BCS (1–5 scale) at calving (L for Low BCS, BCS $\leq$3.5 or H for High BCS, BCS $\geq$3.75; $n = 20$) and (2) feed supplementation with YS (Y for supplemented or C for control; $n = 20$).

All groups had *ad libitum* access to grass silage for the entire 60-day dry period and each group was supplemented with 2 kg/cow/day of a pre-calver concentrate containing thermo-stable (TS) live yeast cultures (2.5 g/cow/day × 10^8 colony forming unit (CFU) of *S. cerevisiae*/g), or 2 kg/cow/day of a control pre-calver concentrate for the last 14 days pre-calving. After calving, cows had *ad libitum* access to a mixture of 50% grass silage and 50% maize silage. Lactating cow concentrate was fed in the milking parlour twice daily beginning with 4 kg/cow/day at calving and increasing stepwise by 0.5 kg/cow/day until a full allocation of 8 kg of concentrate per day was reached. Concentrates for Y group were supplemented with YS (10 g/cow/day × 10^8 CFU of *S. cerevisiae*/g). The live yeast cultures used Yea-Sacc^{1026} TS (Alltech, Inc., Nashville, KY, USA) which was composed of live yeast cells from the strain *S. cerevisiae*^{1026} grown in batch cultures on a media of corn, molasses, malt and trace minerals. The result is a concentrated mixture of live yeast cells and cell metabolites produced during growth. The product is carefully dried to maintain viability (Alltech, Inc.). Ingredient and chemical composition of the feeds used are presented in Tables 1 and 2.

Table 1
Average chemical composition of grass and maize silage used.

Item	Unit	Maize silage	Grass silage
DM	g/kg DM	273	202
CP	g/kg DM	105	140
pH	–	39	40
Ash	g/kg DM	47	56
NDF	g/kg DM	454	483
Starch	g/kg DM	250	–
DMD	g/kg DM	–	745
ME	MJ/kg DM	11.1	10.7
NH_3-N	% of total N	–	7.6

Table 2
Ingredient and chemical composition of the concentrates used.

Item	Pre-partum		Post-partum	
	Control (g/kg as mixed)	Yeast culture (g/kg as mixed)	Control (g/kg as mixed)	Yeast culture (g/kg as mixed)
Ingredient composition				
Wheat	429	429	300.0	300.0
Soya 46%	–	–	348.67	348.67
Double zero rape extracted	–	–	100	100
Soya hulls	–	–	97.0	95.5
Citrus pulp	–	–	50	50
Corn gluten	47.0	44.5	–	–
Palm kernel expeller	88.5	88.5	–	–
Pollard Meneba	150	150	–	–
African cotton extracted	142	142	–	–
Milk solids extracted	78.8	78.8	50	50
Crude palm oil	6.0	6.0	9.28	9.28
Mono-dicalcium phosphate	–	–	4.5	4.5
Lime flour	–	–	7.6	6.5
Salt	3.5	3.5	12.8	12.8
Cal. Mag.	41.1	41.1	12.9	13.0
Vitamin E 5%	9.9	9.9	1.8	1.8
Yea-Sacc T.S. 50% premix	–	2.5	–	2.5
Vitamin/trace element premix[a]	4.2	4.2	5.45	5.45
Volume (kg)	1000	1000	1000	1000
Chemical composition				
DM	882.6	887.2	874.6	883.3

Item	Pre-partum		Post-partum	
	Control (g/kg of DM)	Yeast culture (g/kg of DM)	Control (g/kg of DM)	Yeast culture (g/kg of DM)
Chemical composition				
Ash	105.8	103.7	94.4	95.3
NDF	250.7	262.1	203.4	202.4
ADF	115.1	119.2	130.7	127.2
GE (MJ/kg DM)	17.53	17.54	18.05	17.87
EE	24.5	23.0	23.0	23.4

[a] Contains in prepartum concentrate (dry matter basis): vitamin A, 8500 IU/kg; vitamin D3, 2400 IU/kg; vitamin E, 500 IU/kg; magnesium 2.20%; copper (as CU sulphate), 142.00 mg/kg; copper (as CU chelate of amino acids), 40.00 mg/kg; selenium (as Na selenite), 2.90 mg/kg; iodine (as Ca iodate), 31.00 mg/kg. Contains in post-partum concentrate (dry matter basis): vitamin A, 7000 IU/kg; vitamin D3, 2000 IU/kg; vitamin E, 100 IU/kg; magnesium, 0.85%; copper (as CU sulphate), 37.00 mg/kg; copper (as CU chelate of amino acids) 15.00 mg/kg; selenium (as Na selenite), 0.65 mg/kg; iodine (as Ca iodate), 10.00 mg/kg.

2.3. Feed sampling and analyses

Feed intake was monitored and recorded automatically from day 14 pre-partum until day 70 PP for each animal using the automated feed intake recording system. Concentrate feeding was controlled and measured by the same system during the dry period and in the milking parlour for the milking cows. Samples of the pre-partum and PP concentrates, maize and grass silage were collected weekly and stored at −20 °C prior to analysis. The dry matter (DM) content of maize silage and grass silage was determined by oven drying at 55 °C for 72 h. The concentrate and the dried maize and grass silage samples were milled through a 1 mm screen (Christy and Norris hammer mill), dried at 105 °C for a minimum of 16 h to determine the residual DM in the milled sample, and ashed at 550 °C for 4 h in a muffle furnace (Nabertherm, Bremen, Germany) to determine the ash content. The crude protein content was determined as $N \times 6.25$ using the LECO FP 528 instrument (Leco Instruments, UK Ltd., Newby Road, Hazel Grove, Stockport, SK7 5DA, and Cheshire). The neutral detergent fibre (NDF) and the acid detergent fibre (ADF) content of the concentrate and the NDF content of the maize and grass silage were measured according to the procedures of Van Soest et al. (1991) using a Fibertec extraction unit (Tecator, Sweden). The gross energy (GE) content of concentrate was determined using a Parr 1201 oxygen bomb calorimeter (Parr, Moline, Illinois, USA). The ether extract (EE) was measured using a Soxtec instrument (Tecator). Silage pH was measured by analyzing the juice pressed from the silage using a glass electrode and a pH meter. Determination of *in vitro* dry matter digestibility (DMD) of the grass silage samples was carried out using the procedure described by Tilley and Terry (1963). The starch content of the maize silage was determined using the Megazyme total starch assay kit (Megazyme International Ireland Ltd., Bray Business Park, Bray Co. Wicklow) according to the first action method 996.11 of the AOAC (2001). Ammonia nitrogen (NH_3-N) in grass silage was determined by a modification of the phenol/hypochlorite technique as described by O'Keefe and Sherrington (1983).

2.4. Milk recording, sampling, and analyses

Cows were milked twice daily, and milk yield was recorded at each milking for individual cows. Milk samples were collected weekly at consecutive a.m. and p.m. milkings and the samples were pooled in proportion to their am and pm milk yield. These composite milk samples were kept preserved (Broad Spectrum Microtabs®, D & F Control Systems, Inc.California 94568, USA) and refrigerated until analyzed for fat, protein, lactose, and SCC by near infrared spectrophotometry Milk-O-Scan (Foss Electric, Denmark).

2.5. Body condition score and weight

Body condition score was determined on a five-point scale (1 = emaciated, to 5 = obese) with quarter-point increments, functioning as a 17-point scale (Edmonson et al., 1989). Body weight was assessed weekly starting at calving until 10 weeks PP following the morning milking. Body condition scoring was always performed by the same trained operative by palpating and visualising individual body parts of the spinal column (chine, loin, and rump), the cranial coccygeal vertebrae (tail head), the tuber ischia (pin bones), the tuber sacral (hip or hook bones), and the thigh region (Edmonson et al., 1989). Body weight was measured using a calibrated electronic weighing scale (Tru-Test Ltd., Auckland, New Zealand). The weighing scale was calibrated weekly with known weights.

2.6. Energy balance calculation

Estimated EB during the PP period was calculated on a weekly basis for individual cows as the differences between NE intake and the sum of NE for maintenance and milk production according to Jarrige (1989), as described by McNamara et al. (2003). The NE intake was determined by multiplying the DMI of each feedstuff by its energy value (UFL; Unité Fourragère Lait–Feed Unit for Milk). One UFL is the NE content (1700 kcal NEL) supplied by 1 kg of air-dry standard barley for milk production (Jarrige, 1989). The contribution of concentrate offered in NE intake was determined using the NE values UFL/kg DM of ingredients (INRAtion, 1989). The NE content UFL/kg DM of grass silage was related to its *in vitro* DMD (O'Mara et al., 1997) and of maize silage was related to its starch content.

The following equations were used to estimate the NE requirement for maintenance and milk production:

$$\text{Maintenance NE requirement}:$$

$$\text{UFL/day} = 1.4 + 0.6\,\text{BW}/100$$

A further 10% was added for an activity allowance (Jarrige, 1989).

$$\text{Milk NE requirement}:$$

$$\text{UFL/kg of milk} = 0.054F\% + 0.031P\% + 0.028L\% - 0.015$$

where $F\%$ = milk fat percentage, $P\%$ = milk protein percentage and $L\%$ = milk lactose percentage. Adapted from Tyrrell and Reid (1965).

2.7. Blood sampling and analyses—insulin and insulin-like growth factor-I (IGF-I)

Blood samples (10 ml) were collected by jugular venipuncture from each cow on approximately days 14 and 7 pre-calving, and 1, 5, 15, 25 and 35 PP. Blood was collected into plain evacuated serum tubes (BD Vacutainer Systems, Preanalytical Solutions, Belliver Industrial Estate, Plymouth, PL6 7BP, UK). Blood tubes were kept lying on their side overnight at 4 °C, then, serum was separated by centrifugation at 1600 × g at 4 °C for 15 min and stored frozen at −20 °C until assayed for insulin and IGF-I. Insulin concentration was quantified in serum samples by using a solid-phase fluoroimmunoassay (AutoDELFIA, PerkinElmer Life and Analytical Science, Turku, Finland). All serum samples were analyzed for insulin in a single assay and the intra-assay coefficient of variation (CV) was 3.6%. Serum IGF-I concentrations were determined by using a validated double-antibody radioimmunoassay after ethanol–acetone–acetic acid extraction. The standard and iodinated tracer used was recombinant human IGF-1 (R&D Systems Europe, Abingdon, UK). Iodine-125 (PerkinElmer; Unitech BD Ltd., Dublin, Ireland) was used for the iodination. The extraction and assay were carried out as previously described by Beltman et al. (2010) with inter- and intra-assay CV (n = 4) of 10.76% and 13.23%, respectively.

2.8. Reproductive measurements

Ovarian follicular activity of all cows was examined daily by ultrasonography using an Aloka SSD-500V ultrasound instrument fitted with a 7.5-Mhz linear-array trans-rectal transducer (Aloka Co. Ltd., Tokyo, Japan), beginning on day 10 PP and continuing until ovulation, or until day 70 PP for cows failing to ovulate. Ovarian ultrasonography was used to monitor the size and development of the first dominant follicle and first ovulatory follicle PP and days to first ovulation. Follicle diameter, which was obtained by averaging two perpendicular measurements of the diameter of the follicle determined ultrasonographically, and the number of ovarian follicles (≥3 mm) were recorded onto follicular maps. Follicles were considered to be dominant when a diameter of >10 mm was reached in the absence of other large, growing follicles (Savio et al., 1990). Cysts were defined as anovulatory follicles >25 mm in diameter that persisted for at least 10 day in the absence of a corpus luteum (Garverick, 1997).

The day of ovulation was defined as the day on which a large dominant follicle (DF), present on either ovary the previous day, had disappeared and followed by the formation of a corpus luteum. Jugular blood samples were collected into plain serum tubes (BD Vacutainer Systems) daily at the time of scanning. Blood tubes were kept lying on their side overnight at 4 °C, then, serum was separated by centrifugation at 1600 × g at 4 °C for 15 min and stored frozen at −20 °C until assayed. Serum oestradiol concentrations were determined for the samples collected during the 2 days before ovulation day, to monitor the peak concentrations of the hormone in each treatment. The concentration of oestradiol (E2) in serum was quantified by radioimmunoassay following extraction using E2 MAIA kits (Estradiol Maia, Adaltis Italia S.P.A., Casalecchio di Reno, BO, Italy) as previously described (Prendiville et al., 1995), in two assays with inter- and intra-assay CV (n = 2) of 12.98% and 12.48%, respectively.

2.9. Statistical analyses

All data were checked for adherence to the normal distribution and homogeneity of variance using histograms and formal statistical tests as part of the UNIVARIATE procedure in (SAS v 9.1, 2004). The natural logarithm transformation of insulin and IGF-I were used to normalize the distributions as preliminary analyses revealed that the distribution of values for these analytes was positively skewed. The transformed data were used to calculate *P*-values. However, the exponential values of least squares means and standard errors are presented in the results for clarity. Single time point data (reproductive variables) were analyzed using Mixed procedure in SAS v 9.1 (2004) with terms included for BCS (L or H), YS supplementation (Y or C), block and their interactions included in the model. Variables having more than one observation per subject (NE intake, EB, Insulin and IGF-I) were analyzed using repeated measures ANOVA Mixed procedure in (SAS v 9.1, 2004) with terms for BCS, YS supplementation, block, time period and their interactions included in the statistical model. Cow was the repeatedly studied subject and was modeled as a random effect. Actual calving day and the length of time on the pre-calving diet were used as covariates. The compound symmetry, unstructured and first-order autoregressive variance–covariance structures were tested and the most appropriate (lowest Akaike's information criterion, Akaike's information criterion with correction, and Bayesian information criterion values) was used for each analysis. PROC REG procedure of (SAS v 9.1, 2004) was used for linear regression. The interaction term if not statistically significant ($P > 0.10$) was subsequently excluded from the final model. Accordingly, there were no significant ($P > 0.10$) two-way interactions (BCS × YS) or three way interactions (BCS × YS × time) for any of the measured variables and these were therefore excluded from the data set. As a result, the main effects of BCS at calving and dietary YS supplementation were presented separately. A *P*-value of <0.05 was considered significant and of >0.05 < 0.10 was considered a tendency. Results are presented as least squares means ± standard error of mean SEM, unless otherwise stated.

3. Results

3.1. Effect of BCS at calving

The effect of BCS at calving on NE intake, milk NE output, and EB during the PP period is presented in Fig. 1. Net energy intake of cows in the H group was lower ($P < 0.05$) than for those in the L group in the first and second week PP (13.1 ± 0.49 UFL/day vs. 14.5 ± 0.50 UFL/day and 16.0 ± 0.49 UFL/day vs. 17.4 ± 0.50 UFL/day, respectively). There was a time effect ($P < 0.01$) on NE intake during the PP period; cows in both BCS groups started with the lowest NE intake immediately after calving and then increased by time post-calving (Fig. 1a). Milk NE output for the cows in the H group was higher ($P < 0.05$) than for those in the L group (13.2 ± 0.39 UFL/day vs. 12.3 ± 0.39 UFL/day, respectively) for the overall PP period. Consistently, cows in the H group tended to have higher ($P = 0.07$) milk NE output in the first week PP and significantly higher ($P < 0.05$) milk NE output during week 2, 4, 8 and 9 PP (Fig. 1b). The cows in the H group had lower ($P < 0.01$) EB than those in the L group (−1.7 ± 0.44 UFL/day vs. 0.4 ± 0.43 UFL/day, respectively) during the overall PP period. For each of the first 10 weeks PP the cows in the H group had lower ($P < 0.05$) EB than those in the L group except in week 7 PP ($P > 0.10$; Fig. 1c). There was a time effect ($P < 0.01$) on EB; the cows in all groups started with the lowest EB at the first week PP and then increased with time after calving. From the 4th week PP the cows in the L group entered positive EB status, while those in the H group entered positive EB in the 10th week PP (Fig. 1c).

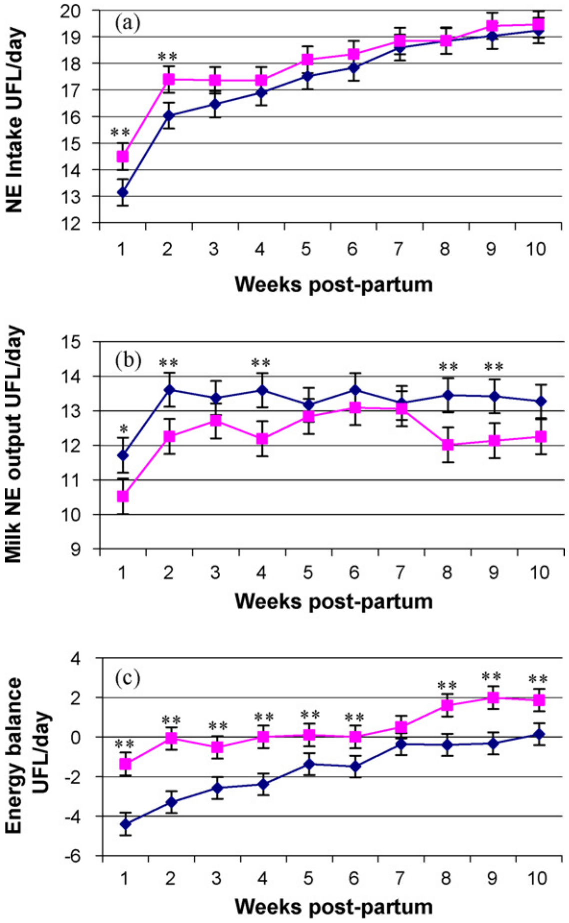

Fig. 1. Effect of BCS at calving (♦ = high BCS, ■ = low BCS) on (a) NE intake, (b) milk NE output and (c) energy balance during the first 10 weeks post-partum. The error bars correspond to the SEM (*$P > 0.05 < 0.10$, **$P > 0.01 < 0.05$, ***$P < 0.01$).

The effect of BCS at calving on insulin and IGF-1 concentrations during the pre- and post-calving period is presented in Fig. 2. During the pre-calving period, insulin concentration was higher ($P < 0.01$) for the cows in the H group in comparison with those in the L group (49.4 ± 1.10 pmol/L vs. 32.3 ± 1.10 pmol/L, respectively). Conversely, during the PP period, the insulin concentration of the cows in the H group was lower ($P < 0.05$) than those in the L group, Fig. 2a (24.6 ± 1.07 pmol/L vs.

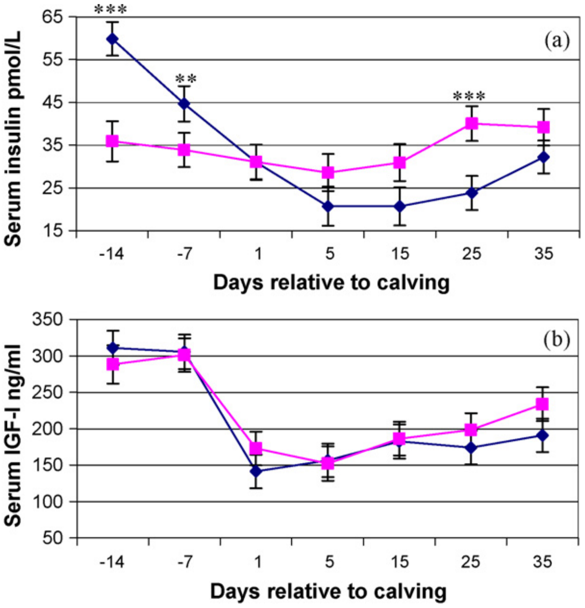

Fig. 2. Effect of BCS at calving (♦ = high BCS, ■ = low BCS) on (a) serum insulin concentration and (b) serum IGF-I concentration during the periparturient period. The error bars correspond to the SEM (*$P>0.05<0.10$, **$P>0.01<0.05$, ***$P<0.01$). Values presented in this figure are exponential of log-least squares means.

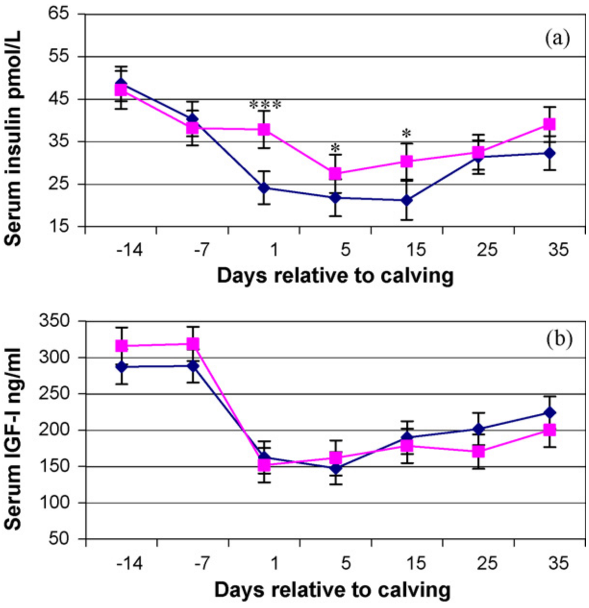

Fig. 3. Effect of live yeast culture (Yea-Sacc^{1026}) supplementation (■ = supplemented, ♦ = control) on (a) serum insulin concentration and (b) serum IGF-I concentration during the periparturient period. The error bars correspond to the SEM (*$P>0.05<0.10$, **$P>0.01<0.05$, ***$P<0.01$). Values presented in this figure are exponential of log-least squares means.

31.0 ± 1.07 pmol/L, respectively). On day 25 PP, insulin concentration was higher ($P<0.01$) for the cows in the L group in comparison with the H group cows (39.8 ± 1.12 pmol/L vs. 23.2 ± 1.12 pmol/L, respectively; Fig. 2a). There was no effect ($P>0.10$) of BCS at calving on the concentrations of IGF-1 during pre- or post-calving period.

Cows in L group tended to have higher ($P=0.06$) pre-ovulatory peak oestradiol concentrations (Fig. 4) during the whole experimental period. There were no significant differences between cows in the H and L groups on days to first PP ovulation ($P>0.10$) averaging 30 ± 4.5 days, the size of the first ovulatory follicle ($P=0.10$) averaging 18.8 ± 0.50 mm, number of follicular waves to first ovulation ($P>0.10$) averaging 2.25 ± 0.43 waves, and the size of first PP dominant follicle ($P>0.10$) averaging 17.55 ± 1.30 mm.

3.2. *Effect of yeast culture supplementation*

There was no effect ($P>0.10$) of feed supplementation with YS on NE intake, NE milk output and EB during the 10 weeks PP. The effect of YS supplementation on serum concentrations of insulin and IGF-I during the pre- and post-calving period is presented in Fig. 3. Serum insulin concentrations were higher ($P<0.01$) for supplemented cows in comparison with the control cows (31.0 ± 1.07 pmol/L vs. 24.6 ± 1.07 pmol/L, respectively) during the overall PP period (Fig. 3a). There was no difference ($P>0.10$) between the concentrations of serum IGF-I during pre- or post-calving periods in the cows in the Y and C groups (Fig. 3b).

The size of the first PP ovulatory follicle tended to be higher ($P=0.09$) in the supplemented cows in comparison with the control cows (19.4 ± 0.51 mm vs. 18.3 ± 0.48 mm, respectively). The pre-ovulatory peak oestradiol concentrations tended to be higher ($P=0.07$) in the Y cows than the C cows averaging 3.3 ± 0.21 pg/ml vs. 2.8 ± 0.19 pg/ml, respectively (Fig. 4). There were no significant differences ($P>0.10$) between Y and C group cows in the days to first PP ovulation averaging 30 ± 4.35 day, the number of follicular waves from parturition until ovulation averaging 2.3 ± 0.42 waves, and the size of the first PP dominant follicle averaging 17.55 ± 1.02 mm.

For all serum samples collected from all cows across treatments, the pre-ovulatory peak in oestradiol concentrations was related to the size of ovulatory follicle

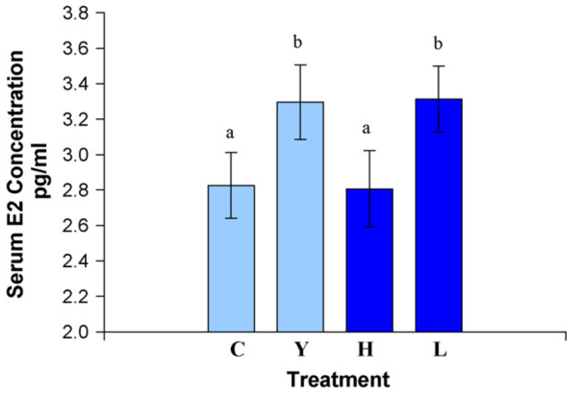

Fig. 4. Effect of live yeast culture (Yea-Sacc^{1026}) supplementation and BCS at calving (C = control, Y = supplemented, H = high BCS, L = low BCS) on pre-ovulatory serum oestradiol peak. The error bars correspond to the SEM. Columns with different letters tended to be different ($P>0.05<0.10$).

($r^2 = 0.35$; $P < 0.01$). When data were pooled across treatments, the mean insulin concentration was related to the mean EB ($r^2 = 0.22$; $P < 0.05$) during the first 4 weeks PP. Likewise, mean insulin concentrations during the first 4 weeks PP was related to the EB during week one PP ($r^2 = 0.44$; $P = 0.001$) and, week two PP ($r^2 = 0.25$; $P = 0.01$).

4. Discussion

Changes in EB during the PP period followed the pattern observed by Villa-Godoy et al. (1988), Sutter and Beever (2000) and McNamara et al. (2003) where an initially severe NEB improved as lactation progressed reaching positive EB by day 50–80 PP on average. Pedron et al. (1993) found that the recovery of BCS (reflected by EB) started at 10 weeks PP for cows in BCS 3 and 3.5 but was delayed until 12 weeks PP for those in BCS 4. In the present study the H group did not recover from NEB until week 10 PP compared with week 4 PP for the L group. In agreement with previous studies (Ryan et al., 2003; Stockdale, 2004, 2008) cows in the H group ingested less NE, produced more milk NE output, and consequently suffered from more severe NEB during early lactation.

The association observed between blood insulin concentrations and energy status reported here, has been previously observed (Blum et al., 2000; Busato et al., 2002; Reist et al., 2003). Accordingly, cows with low BCS at calving (moderate body condition) had higher insulin concentrations in the PP period and adapted better than cows with a high BCS at calving (over-conditioned) to increased energy requirements after parturition. Sartin et al. (1988) stated that the lower insulin concentrations in high producing dairy cows promote lipid mobilisation during early lactation.

The relationships between the mean insulin concentration of the first 4 weeks PP and the mean EB suggest that insulin concentrations might be a good indicator for energy status in the early PP period. However, it has been well accepted that energy status in the early PP period influences the resumption of reproductive function of dairy cows during early lactation (Osoro and Wright, 1992; Beam and Butler, 1999). Furthermore, Gong et al. (2002) reported that high circulating insulin concentrations during early lactation can advance the first PP ovulation, reduce days to first service and calving to conception interval. The relationship between the size of ovulatory follicle and the pre-ovulatory peak oestradiol concentrations found in the present study might be explained by the stimulatory effect of insulin on differentiation and steroidogenesis of follicular cells (Spicer et al., 1993; Stewart et al., 1995; Gutierrez et al., 1997). This may promote the maturation of the dominant follicle in early lactation, and then ovulation in response to LH surge (Beam and Butler, 1997, 1999).

One of the most interesting observations in this study was the tendency of a higher pre-ovulatory peak oestradiol concentrations associated with higher insulin concentration in L and Y groups in comparison with H and C groups, respectively. Numerous studies on different species in vivo and in vitro indicate that insulin has a direct stimulatory effect on granulosa cell oestradiol production and indirect stimulatory effects via amplification of gonadotrophin action (Silva and Price, 2002; Selvaraju et al., 2003; Butler et al., 2004). Furthermore, Butler et al. (2004) reported a sustained increase in circulating oestradiol in response to insulin treatment on dairy cows with absence of a change in LH pulsatility. They explained this observation by many possible mechanisms as follows: (i) insulin had a direct stimulatory effect on overall ovarian steroid production; (ii) insulin had a direct stimulatory effect specifically on aromatization of androgen to oestrogen; (iii) insulin increased ovarian responsiveness to another circulating factor (such as LH or IGF-I); (iv) insulin had an indirect effect via altering circulating levels of another factor (such as NEFA or IGF-I). Accordingly, the tendency of high pre-ovulatory oestradiol peak of the L and C groups in the present study might be attributed to higher PP insulin concentrations of these groups.

It has often been suggested that the body condition score at calving and the energy balance status are some of the most important factors affecting the reproductive performance (Osoro and Wright, 1992; Beam and Butler, 1999; Hoedemaker et al., 2009) of dairy cows. However, the fact that no significant effects of BCS at calving were detected in the current study on the days to first ovulation PP and some of other measured reproductive parameters, has to be interpreted cautiously because of the relatively low number of animals per treatment to assess such fertility indices. However, it is interesting to note that Ruegg and Milton (1995) found that BCS at calving was not a significant source of variation in days to first recorded oestrus, days to first breeding, number of AI services per cow, or days to conception in Holstein dairy cows.

Several studies have reported a significant effect of YS supplementation on lactation performance (Wang et al., 2001; Bruno et al., 2009b; Moallem et al., 2009), possibly due to a positive effect on feed intake, rumen stability and nutrient digestibility (Wohlt et al., 1998; Dann et al., 2000; Denev et al., 2007). The mode of action by which the addition of YS may improve feed efficiency of dairy cows is unknown. Enhanced dry matter, fibrous fractions and crude protein digestibility in the rumen (Wohlt et al., 1998; Miller-Webster et al., 2002) by improving microbial growth in the rumen (Erasmus et al., 1992) and improving the stability of the rumen environment (Callaway and Martin, 1997) by enhancing the growth of lactic acid-utilising bacteria (Denev et al., 2007), may all be possible explanations for increased feed efficiency due to YS supplementation. The lack of significance in the effect of supplementing YS on energy intake reported here was in contrast with the findings of Robinson and Garrett (1999), Erasmus et al. (1992), and Moallem et al. (2009) who found that supplementing diets with YS increased DMI. In the present study the lack of effect on NE intake of adding YS might explain the lack of detectable effect on the milk NE output which is in agreement with other recent investigations (Bruno et al., 2009b; Shwartz et al., 2009).

There are only limited studies on the effect of YS feeding on blood insulin and IGF-I concentrations in dairy cows and none on the effect of YS on these parameters in the immediate PP period. Recently, Bruno et al. (2009b) in their trial with Holstein/Friesian dairy cows, reported no effect of YS addition on insulin concentrations later in the lactation when the most severe NEB has passed (days 58 and 100 PP). Although no significant differences in the NE intake were observed in the present study, YS supplementation increased serum insulin concentration. The reason for this is not clear, as YS did not improve NE intake or energy status. However, Oba and Allen (2003) found that the infusion of propionate in appropriate amounts in the rumen of dairy cows elevated glucose and insulin concentrations. Therefore, the increased insulin concentrations due to YS supplementation shown in the present study may be related to some changes in rumen fermentation.

To our knowledge; only a few studies investigated the effect of supplementing YS on reproductive performance of early lactating dairy cows. Dann et al. (2000) indicated no effect of YS addition on days to first breeding or services per pregnancy of Jersey dairy cows. Bruno et al. (2009a) reported that adding YS to the diet of Holstein cows under heat stress from day 20 to 140 PP, did not influence the proportion of cows that resumed estrous cyclicity by 51 days PP, conception rates, or pregnancy losses. Lehloenya et al. (2008) reported no effect of YS supplementation on days to first and second ovulation early PP.

5. Conclusions

In agreement with previous studies, cows with high BCS at calving (over-conditioned) ingested less NE, had lower concentrations of insulin, produced more milk NE output, and consequently had a longer NEB period than cows with a low BCS at calving (moderate body condition). There was no effect of BCS at calving on ovarian parameters. However, cows with low BCS did exhibit a trend of higher pre-ovulatory peak oestradiol concentrations which was associated with higher insulin levels. Supplementing the diet of lactating dairy cows with live yeast culture *S. cerevisiae*^{1026} had no effect on energy status but did increase serum insulin concentration and tended to elevate pre-ovulatory peak oestradiol concentrations and the size of ovulatory follicle early PP.

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The cryoprotective effects of ascorbic acid supplementation on bovine semen quality

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ABSTRACT

To investigate the effects of ascorbic acid supplementation on standard semen quality parameters and antioxidant activities after thawing of bovine frozen semen, antioxidant ascorbic acid was added at concentrations of 2.5, 4.5, 6.5 and 8.5 mg/ml to bovine semen cryoprotective medium. The results showed that the sperm motility and motion characteristics were improved in the presence of ascorbic acid in extender, as compared to the control. The motility and straight linear velocity (VSL), linearity index (LIN), average path velocity (VAP), wobble coefficient (WOB), lateral head displacement (ALH) values and the percentage of “grade a” sperm in the extender supplemented with 4.5 mg/ml ascorbic acid were significantly higher than that of other treatment groups ($P < 0.05$). The acrosome integrity and membrane integrity were significantly improved ($P < 0.05$) by supplementing with 4.5 mg/ml ascorbic acid in the extender compared with a control. The extender supplemented with ascorbic acid did not lead to any improvement in superoxide dismutase (SOD) levels. The catalase (CAT) activity was higher in the extender supplemented with ascorbic acid at 4.5 mg/ml, when compared with other groups ($P < 0.05$) and the extender supplemented with ascorbic acid significantly decreased glutathione peroxidase (GSH-Px) activity, whereas reduced glutathione (GSH) activities were significantly enhanced, compared with the control ($P < 0.05$). Increasing the doses level of ascorbic acid decreased GSH-Px and GSH activity, the supplementation of 8.5 mg/ml ascorbic acid produced the lowest level of GSH-Px and GSH activity among groups ($P < 0.05$). The extender supplemented with ascorbic acid could reduce the oxidative stress provoked by freezing–thawing and improve bovine semen quality. The particular properties of ascorbic acid are poorly related to its effectiveness in membrane cryopreservation. Further studies are required to determine lipid peroxidation and antioxidant capacities of ascorbic acid in cryopreserved bovine semen.

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1. Introduction

Mammalian sperm membranes are rich in unsaturated fatty acids and lack a significant cytoplasmic component containing antioxidants, and therefore are particularly susceptible to lipid peroxidation (LPO) in the presence of reactive oxygen species (ROS), with subsequent loss of membrane integrity, impaired cell function and decreased sperm motility (Lenzi et al., 2002; Bucak et al., 2007). Under natural systems of mating, sperm cells are exposed primarily to anaerobic conditions, thus reducing potential damage by the ROS. In addition, oviduct fluids contain substantial

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concentrations of taurine (Miller and Shultz, 1987), an important protector of sperm cells against accumulation of ROS when they are exposed to aerobic conditions (Alvarez and Storey, 1992). However, sperm cells are exposed to oxygen and visible light radiation during processing of freezing; these factors can lead to the formation of ROS and increase sperm damage to motility and reduce genomic integrity (Bilodeau et al., 2001; Baumber et al., 2003). Direct and indirect evidence had indicated that some steps of cryopreservation of semen involved the production of toxic ROS (Wang et al., 1997; Bilodeau et al., 2000). In this respect bovine sperm membranes were observed to be sensitive to free radicals (Schiller et al., 2003), and often introduced an additional source for ROS attack on sperm during cryopreservation (Brouwers and Gadella, 2003).

The production of ROS is a normal physiological event in various organs. However, the over-production of ROS causes structural damage of sperm membranes (De Lamirande et al., 1997). To counteract the destructive effects of ROS, seminal plasma has an antioxidant system that seems to be very relevant to the protection of sperm. The sperm oxidative defence enzymes predominantly include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH) and small molecular antioxidants such as ascorbic acid and α-tocopherol (Aitken and Baker, 2004; Sikka, 2004). Mammalian sperm membranes are particularly rich in polyunsaturated fatty acids (PUFA) (Aitken et al., 1993), and this antioxidant capacity in sperm cells may be insufficient in preventing ROS during the freezing–thawing process.

To control the level of ROS and promote motility and survival of sperm, numerous antioxidants have proven beneficial in treating male infertility (Sinclair, 2000). Ascorbic acid is not only effective as a non-enzymatic antioxidant but also water-soluble ROS scavenger with high potency (Silva, 2006). Ascorbic acid is also a naturally occurring free radical scavenger and as such its presence assists various other mechanisms in decreasing numerous disruptive free radical processes, including LPO (Knight et al., 1993).

The addition of ascorbic acid in an extender can impact on optimal sperm performance by reducing cell damage through its continuous radical-scavenging action. Beconi et al. (1993) had indicated that the presence of 5 mM ascorbic acid in the freezing diluent exerted an antioxidant effect during freezing and thawing of bovine sperm. Sierens et al. (2001) demonstrated that antioxidant species might act in vivo to decrease oxidative damage to DNA, protein and lipids, and this finding suggested that ascorbic acid might be needed to protect sperm against reactive oxygen species. Ascorbic acid is a non-enzymatic antioxidant and, therefore is, potentially involved in protecting cells against oxidative stress (Anane and Creppy, 2001).

With the increasing evidence that ROS can potentially damage genomic material and sperm membranes, it is important to determine if any improvement in survival and fertility of cryopreserved bull sperm can be accomplished by supplying semen extender with various antioxidants. Despite investigations on the effects of antioxidants on female and male gametes, and embryo development, little is known of the effects of the addition of ascorbic acid on the post-thaw bovine semen quality and sperm antioxidant activities (SOD, CAT, GSH-Px and GSH). Therefore, the objective of this study was to investigate the effects of addition of ascorbic acid to a semen extender on standard semen quality parameters (sperm motile characteristics, acrosome integrity and membrane integrity) and antioxidant enzymes activities (SOD, CAT, GSH-Px and GSH), following the freezing–thawing process.

2. Materials and methods

Unless otherwise stated, all chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Semen collection and processing

Bovine semen was collected by artificial vagina from six bulls at the Domestic Animal Improving Station (Shaanxi Province, China), and two ejaculates were obtained from each bull and the semen was held in a water bath at 35 °C while the sperm concentration and initial percentage of motile sperm were being estimated. Sperm concentration was estimated by optical density using a calibrated spectrophotometer. The percentage of motile sperm was estimated at 37 °C by light microscopy. The ejaculates with >75% motility and >85% normal sperm morphology were used for the present study. Consequently, 12 ejaculates from six bulls were used. The semen was pooled and apportioned between the different extenders (the treatments and control).

The cryoprotective extender for the treatments used in this study was composed of 2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.6 ml glycerol, 20 ml egg yolk, 25 mg gentamicine, 50,000 UI penicillin for 100 ml deionized water (Packer et al., 1979), and supplemented with different concentrations of ascorbic acid (2.5, 4.5, 6.5 and 8.5 mg/ml). The cryoprotective extender for the control group was no supplementation with ascorbic acid, which was the only difference with extender for the treatments. In this study ascorbic acid (Vitamin C, the highest commercially available purity) liquid supplement was used and the net content was 0.5 g/5 ml. It was made by China NingXia Pharmaceutical Co., Ltd.

After the evaluation of quality, the fresh semen was then divided into five equal fractions, one fraction was diluted with the extender for the control group (no ascorbic acid), and others with the extender for the treatments (2.5, 4.5, 6.5 and 8.5 mg/ml ascorbic acid) to obtain 120×10^6 sperm/ml. Semen was cooled from 37 °C to 4 °C for 1.5 h. PVC straws (0.25 ml) (Biovet, France) were filled and maintained at 4 °C for 2.5 h. The straws were then placed 3 cm above the liquid nitrogen surface and were cooled at approximately −15 °C/min from +4 °C to −120 °C. These straws were then transferred to a liquid nitrogen tank (−196 °C) for storage for at least 2 weeks before thawing.

2.2. Analysis of standard semen quality parameters

The characteristics of sperm motion were assessed using the WLJY-9000, a computer-aided sperm analysis

(CASA) system (WeiLi Software Co. Ltd, Beijing, China). For each extender, three straws were thawed separately by immersion in a water bath at 37 °C for 45 s. The thawed semen samples were immediately transferred into 1 ml plastic tubes and incubated at 37 °C for 10 min. Five microliters of each straw was examined and six fields were randomly chosen. The median values of sperm motility (% of motile sperm), the straight line velocity (VSL, μm/s), the curvilinear velocity (VCL, μm/s), amplitude of the lateral head displacement (ALH, μm), velocity of the average path (VAP, μm/s) and frequency of head displacement (BCF, Hz) were obtained from the video recording. The linearity index (LIN = (VSL/VCL) × 100), mean coefficient (STR = (VSL/VAP) × 100) and wobble coefficient (WOB = (VAP/VCL) × 100) were analyzed. At the same time, sperm were classified by CASA as “grade a”, “grade b”, “grade c” and “grade d”. They were described as follows: rapidly progressive (grade a), slowly progressive (grade b), locally motile (grade c: flagellating but non-progressive motion) and immotile (grade d).

Acrosome integrity was evaluated using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA, 100 μg/ml) in a procedure slightly modified from the method described by Aboagla and Terada (2003). The acrosome status of the sperm was examined using an epifluorescence microscope (LEIKA DM-IRB linked up to a Nikon digital camera DXM). The whole acrosome was visualized with strong green fluorescence under a fluorescence microscope and was scored as acrosome-intact sperm cells. The percentage of fluorescent acrosome-intact sperm was counted in at least 300 sperm cells per slide.

Plasma membrane integrity was evaluated by the hypotonic swelling test (HOST). The straws were thawed in a water bath at 37 °C for 45 s. The 50 μl semen was added to 1 ml of a hypoosmotic solution prepared with 7.35 g sodium citrate and 13.51 g fructose in 1000 ml distilled water. After incubation for 60 min at 37 °C, sperm swelling was assessed by placing 15 μl of well-mixed sample on a warm slide (37 °C) under light microscopy at 400× magnification. Viable sperm had coiled tails after HOST. At least 300 sperm per slide were observed. The sperm were classified as positive or negative based on the presence or absence of coiled tail.

2.3. Biochemical assay

The volume of 120 μl frozen-thawed semen was centrifuged at 25 °C for 5 min at 1600 × *g* after freezing. After elimination of the supernatant, 360 μl 1% Triton X-100 was added into the precipitate to extract for 20 min, subsequently centrifuged at 25 °C for 30 min at 4000 × *g*. The precipitate was suspended once again. Then the supernatant were collected as the crude extract of enzyme in the semen. The enzymatic activities of SOD, CAT, GSH and GSH-Px were detected using a kit (NanJing JianCheng Co. Ltd., China).

The SOD activity was measured using the method of Flohe and Otting (1984). Briefly, each semen sample (frozen-thawed semen) was diluted 1:5 with phosphate buffered sodium (50 mM, pH 7.0). The assay solution contained sodium-carbonate buffer (50 mM, pH 10.0), 0.1 mM xanthine, 0.025 mM nitroblue tetrazolium, 0.1 mM EDTA, xanthine oxidase and sample were mixed in a small cuvette. One unit of SOD activity was defined as the amount of enzyme, which required to inhibition of nitroblue tetrazolium. The SOD activity was measured at 560 nm on the spectrophotometer (Shanghai Spectrophotometer Co. Ltd., China) and expressed as U/ml.

The CAT activity was measured according to the method of Goth (1991). Briefly, 0.3 ml of semen samples (frozen-thawed semen) was incubated in 1.7 ml substrate (65 μM hydrogen peroxide in 50 mM phosphate buffer, pH 7.0) at 37.5 °C for 60 s. The enzymatic reaction was terminated with 1.0 ml of 32.4 mM ammonium molybdate. The hydrogen peroxide was measured at 405 nm against blank containing all the components except the enzyme on a spectrophotometer (Shanghai Spectrophotometer Co. Ltd., China). The values of CAT activity were expressed as U/ml.

The GSH-Px activity was determined using the method described by Lawrence and Burk (1976). The composition of reaction mixture were as follows: 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM reduced NADPH, 1 EU/ml oxidized glutathione reductase, 1 mM GSH, and 0.25 mM H_2O_2. Before the initiation of the reaction induced by the addition of 0.1 ml of peroxide solution, an enzyme source (0.1 ml) was added to 0.8 ml of above reaction mixture and was incubated at 25 °C for 5 min. The absorbance at 412 nm was recorded for 5 min on the spectrophotometer (Shanghai Spectrophotometer Co. Ltd., China). The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The GSH-Px activity was expressed as U/l for sperm samples.

The GSH content of sperm was measured using the method of Sedlak and Lindsay (1968). The semen samples (frozen-thawed semen) were precipitated with 50% trichloracetic acid and then centrifuged at 1000 × *g* for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml Tris-EDTA buffer and 0.1 ml DTNB (5,5′-dithiobis-2-nitrobenzoic acid). The solution was incubated at 22 °C for 5 min and then read at 412 nm on a spectrophotometer (Shanghai Spectrophotometer Co. Ltd., China). The values of GSH were expressed as U/l for sperm samples.

2.4. Statistical analysis

All experiments were done using a minimum of ten replicates for each treatment and control group, and the results were expressed as mean values ± S.D. The Kolmogorov–Smirnov test was first utilized for normality analysis of the data. Given a Gaussian distribution of the data, a general linear model procedure was used to evaluate the differences. The mean values of the percentages of motile sperm, acrosome-intact, plasma membrane-intact and enzyme activity were compared using Duncan’s multiple range test after one-way analysis of variance (ANOVA) procedure, when the *F*-value was significant ($P < 0.05$). All analyses were performed by using Statistical Product and Service Solutions (SPSS 11.5 for Windows; SPSS, Chicago, IL, USA).

Table 1
CASA-obtained, mean values of motility parameters from frozen-thawed bovine semen samples in the presence and the absence of ascorbic acid (AA).

Item	Control	AA 2.5 mg/ml	AA 4.5 mg/ml	AA 6.5 mg/ml	AA 8.5 mg/ml
Motility (%)	36.18 ± 1.53^{c}	38.34 ± 1.35^{bc}	50.61 ± 1.62^{a}	42.36 ± 1.83^{b}	35.30 ± 1.37^{c}
VSL (μm/s)	17.29 ± 1.12^{bc}	17.62 ± 0.89^{bc}	25.63 ± 2.38^{a}	19.40 ± 1.17^{b}	16.29 ± 0.96^{c}
VCL (μm/s)	44.14 ± 1.14^{c}	45.37 ± 1.62^{bc}	47.68 ± 1.94^{ab}	49.44 ± 2.21^{a}	48.64 ± 1.76^{a}
LIN (%)	39.57 ± 1.32^{b}	38.75 ± 1.87^{b}	53.53 ± 2.35^{a}	39.24 ± 1.96^{b}	33.31 ± 1.66^{c}
STR (%)	72.31 ± 2.34^{b}	73.49 ± 2.83^{ab}	77.27 ± 3.02^{a}	74.79 ± 2.67^{ab}	64.82 ± 1.98^{c}
VAP (μm/s)	24.59 ± 1.11^{b}	24.07 ± 1.14^{b}	33.17 ± 2.64^{a}	25.94 ± 1.98^{b}	25.75 ± 1.73^{b}
WOB (%)	55.71 ± 2.35^{bc}	52.94 ± 1.88^{c}	69.28 ± 2.31^{a}	52.47 ± 2.07^{c}	52.94 ± 2.24^{c}
ALH (μm)	1.37 ± 0.14^{de}	1.79 ± 0.18^{bcd}	2.29 ± 0.28^{a}	1.91 ± 0.27^{b}	1.60 ± 0.19^{d}
BCF (Hz)	10.68 ± 1.23^{b}	12.31 ± 1.78^{a}	8.21 ± 0.86^{c}	10.34 ± 1.15^{b}	12.55 ± 1.64^{a}

Values are mean ± S.D. of motility parameters from the CASA data set of thawing bovine spermatozoa in different treatments. Values in the same row with different letters means significantly difference ($P < 0.05$).

3. Results

3.1. Sperm motility and movement characteristics

The effects of the supplementation of ascorbic acid in the extender on cryopreservation sperm motion characteristics are shown in Tables 1 and 2. The motility and VSL, LIN, VAP, WOB, ALH values and the percentage of "grade a" sperm in the extender supplemented with 4.5 mg/ml ascorbic acid were significantly higher than that of the control and the other ascorbic acid concentrations ($P < 0.05$), whereas the BCF value was significantly lower ($P < 0.05$). When ascorbic acid concentration increased to 8.5 mg/ml, sperm motion characteristics and the percentage of "grade a" sperm significantly decreased compared with the treatment group of 4.5 mg/ml ascorbic acid ($P < 0.05$).

3.2. Acrosome and membrane integrity

The results of acrosome and membrane integrity of frozen-thawed bovine sperm are shown in Fig. 1. The percentages of acrosome-intact and plasma membrane-intact sperm were significantly improved ($P < 0.05$) by supplementing with 4.5 mg/ml ascorbic acid. However, when ascorbic acid concentration in extender increased to 6.5 mg/ml, the percentages of acrosome-intact and plasma membrane-intact sperm were significantly lower ($P < 0.05$) as compared with the extender supplemented with 4.5 mg/ml. There were no significant difference of

Table 2
CASA-obtained, mean values of the percentage of spermatozoa grade from frozen-thawed bovine semen samples in the presence and the absence of ascorbic acid (AA).

Item	Grade a sperm (%)	Grade b sperm (%)	Grade c sperm (%)	Grade d sperm (%)	Grade a + b sperm (%)
Control	14.26 ± 1.96^{bc}	23.17 ± 1.47^{c}	47.68 ± 3.07^{c}	14.89 ± 1.24^{ab}	37.43 ± 1.73^{c}
AA 2.5 mg/ml	12.94 ± 1.59^{b}	25.29 ± 2.47^{bc}	46.38 ± 2.66^{c}	15.39 ± 0.97^{ab}	38.23 ± 1.86^{bc}
AA 4.5 mg/ml	19.62 ± 2.13^{a}	31.05 ± 3.21^{a}	40.29 ± 2.48^{b}	9.04 ± 0.73^{c}	50.67 ± 2.65^{a}
AA 6.5 mg/ml	13.42 ± 2.08^{bc}	29.08 ± 2.72^{ab}	45.77 ± 2.97^{ac}	11.73 ± 1.01^{bc}	42.50 ± 2.12^{b}
AA 8.5 mg/ml	10.39 ± 1.84^{bc}	24.15 ± 2.14^{c}	48.87 ± 3.16^{ac}	16.59 ± 1.12^{a}	34.54 ± 2.03^{c}

Values are mean ± S.D. of the percentage of sperm grade from the CASA data set of thawing bovine spermatozoa in different treatments. Values in the same row with different letters means significantly difference ($p < 0.05$).

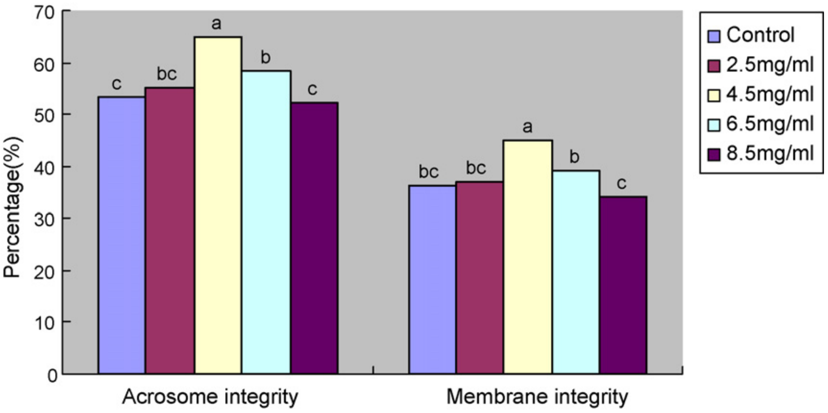

Fig. 1. Microscope observation-obtained, mean values of the acrosome and membrane integrity from frozen-thawed bovine semen samples in the presence and the absence of ascorbic acid (AA). In each set of bars, different letters means significantly difference ($P < 0.05$).

Table 3

Total superoxide dismutase(SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH) levels in frozen-thawed bovine semen in the presence and the absence of ascorbic acid (AA).

Groups	SOD (U/ml)	CAT (U/ml)	GSH-Px (U/l)	GSH (U/l)
Control	1.57 ± 0.11^{a}	2.02 ± 0.15^{b}	166.18 ± 18.67^{a}	19.77 ± 2.07^{d}
AA 2.5 mg/ml	1.36 ± 0.13^{a}	2.08 ± 0.28^{b}	120.12 ± 15.69^{b}	41.80 ± 3.68^{a}
AA 4.5 mg/ml	1.42 ± 0.09^{a}	3.87 ± 0.36^{a}	111.37 ± 14.87^{c}	33.67 ± 3.49^{b}
AA 6.5 mg/ml	1.50 ± 0.07^{a}	2.03 ± 0.45^{b}	110.39 ± 14.59^{c}	24.21 ± 2.52^{c}
AA 8.5 mg/ml	1.46 ± 0.02^{a}	1.39 ± 0.51^{c}	97.64 ± 13.92^{d}	11.28 ± 2.86^{e}

Values are mean ± S.D. of thawed bovine spermatozoa in all the concentration of ascorbic acid. Values in the same row with different letters means significantly difference ($P < 0.05$).

acrosome and membrane integrity between the extenders containing 8.5 mg/ml ascorbic acid and the control group ($P > 0.05$).

3.3. *Analysis of antioxidant activities*

The effects of ascorbic acid supplementation in the extender on antioxidant activities of frozen-thawed bovine semen are shown in Table 3. The extender supplemented with ascorbic acid did not lead to any improvement in SOD levels and there was a slight reduction compared with the control ($P > 0.05$). CAT levels were higher in the group supplemented with 4.5 mg/ml ascorbic acid than the other groups ($P < 0.05$). The extender supplemented with ascorbic acid significantly decreased GSH-Px activity and enhanced GSH activities when compared with the control ($P < 0.05$). Increasing the doses of ascorbic acid decreased GSH-Px and GSH activity while the supplementation of 8.5 mg/ml ascorbic acid resulted in the lowest level of GSH-Px and GSH activity when compared with other groups ($P < 0.05$).

4. Discussion

Low toxicity and good water solubility of ascorbic acid has led to its use as an antioxidant additive and has been shown to have protective effects when added to an extender. The results of this study show that the addition of ascorbic acid to cryoprotective medium increased bovine sperm motility, movement characteristics and the integrity of acrosome and membrane. Addition of 4.5 mg/ml ascorbic acid into an extender suppressed deleterious effect of ROS on bovine sperm. Similar results have been reported by Beconi et al. (1993), Geva et al. (1996), Hsu et al. (1998) and Mirzoyan et al. (2006) that ascorbic acid supplementation reduced ROS generation.

Depending on the concentrations of ascorbic acid, positive as well as negative effects were identified in this study. The extender supplemented with ascorbic acid at the concentration of 4.5 mg/ml resulted in a significant increase in sperm motility and VSL, LIN, VAP, WOB, ALH values and the percentage of “grade a” sperm. Higher ascorbic acid concentrations (up to 8.5 mg/ml) resulted in significant decrease in sperm motion characteristics and the percentage of “grade a” sperm. The percentages of acrosome-intact and membrane-intact sperm were also significantly improved by supplementing with 4.5 mg/ml ascorbic acid, whereas there was a significant decline when ascorbic acid concentration in extender increased to 6.5 mg/ml. Beconi et al. (1993) demonstrated that good quality bull semen appears to benefit more from ascorbate, Vitamin E and SOD than poor quality semen and Foote et al. (2002) reported that higher concentrations of ascorbic acid was detrimental to sperm motility of frozen-thawed bull semen confirming the results of the current study.

In the present study, the extender supplemented with ascorbic acid increased the levels of CAT and GSH activity, while there was a slight reduction in SOD levels. In addition, increasing the doses of ascorbic acid decreased GSH activity. These results indicate that the antioxidant capacity of ascorbic acid could prevent membrane lipid peroxidaion during freezing–thawing and thus protect sperm membranes. Low ascorbic acid levels would allow for physiological production of reactive oxygen species that are essential for the membrane changes (De Lamirande et al., 1997). An inappropriate increase in ascorbic acid concentration could alter plasma membrane features and reduce the possibility of lipid peroxidation, thus affecting its fluidity or the production of the required reactive oxygen species for such processes. The addition of ascorbic acid into the extender could prevent active forms of oxygen generation and membrane lipid peroxidation and, therefore, decrease deleterious effects of cryopreservation. Moreover, important variations in GSH activity values among different ascorbic acid concentrations were found, while variations in SOD activity were less obvious. This is in agreement with previous reports on human sperm which described high variations of GSH level with constant SOD activity values (Alvarez et al., 1987). This was also in agreement with the findings of Bucak et al. (2007, 2008) who demonstrated a marked improvement in ram sperm motility associated with the increased CAT activity of frozen sperm supplemented with the antioxidants taurine and cysteine. These reports and the current findings indicated that antioxidant capacity is not the major factors influencing sperm survival following the freezing–thawing.

5. Conclusion

On the basis of the present results it is concluded that ascorbic acid is a very efficient antioxidant and that its addition to an extender reduces the oxidative stress provoked by thawing and protects bovine sperm during cryopreservation. However, addition of ascorbic acid effectively elevated the levels of CAT and GSH, did not enhance SOD activity and decreased GSH-Px activity. The effectiveness of extender containing ascorbic acid might be related to an improved antioxidant action that provides better membrane protection. More research is needed to understand the precise physiological role of ascorbic acid in sperm protection.

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Study on the inter-subspecies nuclear transfer of river buffalo somatic cell nuclei into swamp buffalo oocyte cytoplasm

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ABSTRACT

The objective of this study was to explore the feasibility of inter-subspecies somatic cell nuclear transfer (SCNT) of river buffalo (50 chromosomes) somatic cell nuclei into swamp buffalo (48 chromosomes) oocyte cytoplasm. The enucleated swamp buffalo oocytes were fused with four different types of river buffalo cells: freshly thawed ear fibroblasts, serum-starved ear fibroblasts, cumulus cells and ear fibroblasts from a cloned buffalo calf. As a result, the developmental competence of embryos reconstructed with freshly thawed ear fibroblasts was the poorest ($P < 0.01$), while those of the other three types were not different from each other. Furthermore, the efficiency of swamp–swamp buffalo, swamp–river buffalo and bovine-buffalo SCNT were also compared. The results showed that the blastocyst rate of swamp–river reconstructed embryos was not different from swamp–swamp embryos, while significantly higher than that of bovine-buffalo embryos ($P < 0.01$). A total of thirty cloned blastocysts derived from freshly thawed ear fibroblasts were transferred into thirteen recipient buffalos, four recipients established pregnancy, while three of them aborted on Days 65, 75 and 90 of gestation, respectively. One cross-bred buffalo (Murrah × swamp, 49 chromosomes) receiving three embryos delivered a 39 kg female calf on Day 335 of gestation. These results indicate that the inter-subspecies SCNT is feasible to produce swamp–river buffalo embryos, and these can develop to full term and result in live buffalo calves.

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1. Introduction

Water buffalo (Bubalus bubalis) is broadly classified into river and swamp subspecies. The swamp buffalo, which is small-sized and mainly raised for work power, has 48 chromosomes. The river buffalo, which is large-sized and primarily used for milk production, has 50 chromosomes. Mitochondrial DNA analyses of water buffalo support a distinct genetic origin of river and swamp buffalos (Kumar et al., 2007). The population of buffalos in China ranks third in the world and are all of swamp type. China has introduced river buffalo to improve the productivity of swamp buffalo, and several reproductive technologies have been adopted with SCNT being a potential valuable tool to increase the number of river buffalos. However, although there are a few reports on SCNT in buffalo (Meena and Das, 2006; Parnpai et al., 2002; Saikhun et al., 2004; Shah et al., 2009; Shi et al., 2007; Simon et al., 2006; Srirattana et al., 2009; Yang et al., 2009), only two live cloned buffalos have been reported (Shi et al., 2007; Yang et al., 2009).

Bovine, rabbit, sheep and goat cytoplasm have been shown to support the development of somatic cell nuclei from various mammalian species (Chen et al., 2002; Dominko et al., 1999; Lanza et al., 2000; Loi et al., 2001; White et al., 1999; Xu et al., 2008), inter-species SCNT was proven successful with the birth of a cloned mouflon

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created by transferring a mouflon granulosa cell into an enucleated sheep oocyte (Loi et al., 2001). Inter-subspecies SCNT has also been reported to be successful in bovine, cat and goat (Gómez et al., 2004; Jian-Quan et al., 2007; Meirelles et al., 2001; Pope et al., 2000; Steinborn et al., 2002). However, attempts to reconstruct animals by inter-species and inter-subspecies SCNT have led to limited success. As foreign mitochondria of somatic cells are introduced into the oocytes, some degree of heteroplasmy can occur after SCNT (Steinborn et al., 2000). The incompatibility of nuclear and mitochondrial encoded components from different species and subspecies is likely to hinder the normal development of reconstructed embryos and affect the efficiency of SCNT. Therefore, it is more feasible to perform SCNT among the closely related subspecies to produce viable offspring. Accordingly, cytoplasm of swamp buffalo is a potential host for somatic cell nuclei from river buffalo.

The main objective of this study was to investigate the ability of somatic cell nuclei of river buffalo to differentiate in swamp buffalo cytoplasm. Furthermore, the feasibility of cell cryopreservation and the SCNT efficiency of ear fibroblasts and cumulus cells were examined, and serial cloning was firstly employed to produce the second-generation nuclear transferred (NT) embryos in buffalo. In addition, the developmental competence of inter-subspecies NT embryos was also compared with that of inter-species embryos.

2. Materials and methods

The petri dishes were produced by Nunc (Wiesbaden, Germany). All chemicals and media used in this study were purchased from Sigma (St. Louis, MO), with the exception of fetal bovine serum (FBS), which was purchased from Gibco BRL (Paisley, Scotland, U.K.).

2.1. *In vitro maturation (IVM) of swamp buffalo oocytes*

Oocytes were recovered from swamp buffalo and bovine ovaries which were transported to the laboratory in normal saline at 25 °C within 2 h from the abattoir. The cumulus–oocyte complexes (COCs) were matured in IVM medium (TCM199 + 10% FBS + 5 μg/mL FSH + 10 μg/mL LH + 0.2 mM sodium pyruvate + 1 μg/mL E_2 + 50 μM cysteine + 25 ng/mL EGF) in a 5% CO_2 incubator at 38.5 °C for 22–24 h. Cumulus cells were removed from oocytes by repeated pipetting after brief exposure to 2 mg/mL hyaluronidase. Oocytes that only had extruded the first polar body were used as cytoplasm for SCNT.

2.2. *Preparation of donor cells*

Several pieces of ear skin were obtained by biopsy from a female river buffalo calf (6 d) and a cloned buffalo calf (15 d), respectively. Tissues were sterilized and manually cut into small pieces, and then enzymatically digested with 0.25% trypsin and 0.05% EDTA at 4 °C for 18–20 h, the preliminary digested tissues were further digested at 37 °C for 40 min. The disaggregated cells were washed 3 times in cell culture medium (DMEM + 10% FBS + 100 IU/mL penicillin + 50 IU/mL streptomycin) by centrifugation at 260 × *g* for 5 min, and cultured under a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

COCs recovered from an abattoir ovary (swamp type) and COCs derived from a river buffalo by ovum-pick-up (OPU) were cultured for 24 h, the cumulus cells were isolated from oocytes by repeated pipetting, then the separated cells were cultured in cell culture medium under a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

The confluent monolayers were obtained and routinely passaged. After three passages, cells were cultured until approximately 70–90% confluent before enzymatic digestion, the digested cells were blended with DMEM supplemented with 10% FBS and 10% dimethyl sulfoxide (DMSO) and transferred into 0.25 mL plastic straws and cell tubes, the straws and tubes were store at −4°C for 2 h and then at −80°C for another 12 h, and cryopreserved in liquid nitrogen. The thawed cells were diluted with DMEM and centrifuged at 260 g for 5 min to remove cryoprotectants, and four types of donor cells were used in this experiment as follows:

Freshly thawed ear fibroblasts: the thawed cells were re-suspended in DMEM and directly used for SCNT.

Serum-starved ear fibroblasts (G0 ear fibroblasts): the thawed fibroblasts were cultured in DMEM supplemented with 10% FBS for 24 h before confluent, and then in DMEM supplemented with 0.5% FBS for another 5 days. The treated cells were trypsinized and re-suspended in DMEM as donors.

Cumulus cells: cells were thawed and treated by serum starvation.

Ear fibroblasts from a clone (G1 ear fibroblasts): the thawed ear fibroblasts derived from a cloned river buffalo calf were serum-starved and digested for SCNT.

2.3. *Enucleation of buffalo oocytes*

The oocytes were placed in 30–40 μL droplets of enucleation medium (TCM199 + 7.5 μg/mL CB + 10% FBS) for 5 min before micromanipulation. The first polar body and metaphase-II plate were removed by aspiration with a bevelled pipette (inner diameter approximately 20–25 μm) under the Nikon TE300 inverted microscope equipped with a micromanipulator (NT-88-V3, Narishige, Tokyo, Japan) and the Spindle-View System (CRI, Woburn, MA, USA).

2.4. *Cell transfer, fusion, activation and embryo culture*

A single fibroblast cell was introduced into the perivitelline space of the enucleated recipient oocyte with a micropipette through the slit in the zona pellucida made during enucleation. Three types of couplets were reconstructed as follows:

Swamp–river couplets: the serum-starved cumulus cells from a river buffalo were transferred into the enucleated swamp buffalo oocytes.

Swamp–swamp couplets: the serum-starved cumulus cells from a swamp buffalo were transferred into the enucleated swamp buffalo oocytes.

Bovine–buffalo couplets: the serum-starved cumulus cells from a river buffalo were transferred into the enucleated swamp buffalo oocytes.

The couplets were washed twice with the embryo culture medium (CM, TCM-199 + 10% FBS), and then equilibrated for 5 min in fusion medium (250 mM sorbitol + 0.1 mM calcium acetate + 0.5 mM magnesium acetate + 0.5 mM Hepes + 0.1% BSA). Electrofusion was performed in a chamber with two electrodes 1 mm apart overlaid with fusion medium. The couplets were manually aligned so that the contact surface between the cytoplast and donor cell was parallel to the electrodes. Cell fusion was induced by 2 consecutive direct current pulses of 1 kV/cm for 10 μs delivered by an ECM2001 manipulator (BTX Inc., San Diego, CA). One hour later, the fused reconstructed embryos were activated by exposing to 5 μM ionomycin in CM for 5 min and followed by a subsequent incubation in CM supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) for 3–4 h.

Following activation, reconstructed embryos were transferred on a buffalo granulosa cell monolayer in a 30 μl droplet of CM overlaid with mineral oil, and cultured under a humidified atmosphere of 5% CO_2 in air at 38.5 °C, the CM was half changed at an interval of 2 days. The cleavage of reconstructed embryos was checked 48 h after culture, and the number of blastocysts was recorded within 8 days of culture.

2.5. Embryo transfer (ET)

The synchronized or natural estrous local and cross-bred buffalos were selected as recipients. The cloned blastocysts were nonsurgically transferred into the uterine horn of recipients ipsilateral to the ovary containing a palpable corpus luteum of recipients at Days 5–8 of the estrous cycle. Each recipient received two or three embryos, pregnancy diagnosis was carried out 30 days after ET by ultrasonography (Aloka SSD-500 scanner with a 5 MHz linear probe, Aloka Co Ltd., Tokyo, Japan), and the pregnancy diagnosis was repeated every 30 days.

2.6. Microsatellite analysis

The donor cells, the nuclear donor buffalo, the cloned buffalo calf, the surrogate mother and one random buffalo (Murrah × swamp) were genotyped using the following 12 primer sets: ETH10, TGLA126, TGLA227, BM2113, ETH225, ETH3, SPS115, ILSTS006, BM1862, BM2934, BM720 and TGLA153. Genomic DNA was extracted from the blood of all the buffalos. The PCR primers for microsatellite markers labeled with fluorescent dyes (9 FAM and 3 HEX) were synthesized by Shanghai Jikang Biotechnology. All multiplexing and loci were analyzed by Genescan v. 3.7 and Genotyper v. 3.7 software (Applied Biosystem, Foster City, CA, USA).

2.7. Statistical analysis

Data were summarized as means ± standard deviation. Statistical analysis was carried out using SPSS11.0 software (SPSS, Chicago, IL, USA) and one-way ANOVA was used to determine significant differences between groups. Probability values less than 0.05 ($P < 0.05$) and 0.01 ($P < 0.01$) were considered to be statistically significant.

3. Results

3.1. Effects of donor cells on SCNT in buffalo

As shown in Table 1, the fusion rate (64.3%) and blastocyst rate (9.5%) of reconstructed embryos from freshly thawed fibroblasts were significantly lower than those from serum-starved fibroblasts (76.8% and 16.2%) ($P < 0.01$). The fusion rate of couplets derived from ear fibroblasts was significantly lower than that from cumulus cells (76.8% vs 82.5%) ($P < 0.05$), while there was no differences in cleavage rate and blastocyst rate between the two groups. The developmental competence of embryos derived from G1 ear fibroblasts was not different from G0 embryos.

3.2. Efficiency of inter-subspecies and inter-species SCNT in buffalo

As shown in Table 2, the cleavage rate of bovine-buffalo NT embryos (77.8%) was significantly higher than those of swamp–swamp (50.9%) and swamp–river NT embryos (56.9%) ($P < 0.01$), while the blastocyst rate of bovine-buffalo NT embryos (5.9%) was significantly lower than those of swamp–swamp (18.8%) and swamp–river NT embryos (17.9%) ($P < 0.01$). The developmental competence of swamp–swamp NT embryos was not different from that of swamp–river NT embryos.

3.3. Pregnancy and calf birth following ET

As shown in Table 3, a total of thirty blastocysts were transferred into thirteen recipient buffalos, two to three blastocysts were transferred into each recipient. Four recipients were confirmed to be pregnant at Day 30 after transfer, while three of the pregnancies aborted on Days 65, 75 and 90 of gestation, respectively. One cross-bred buffalo (Murrah × swamp buffalo) receiving three embryos delivered a female Murrah calf weighing 39 kg on Day 335 of gestation, the cloned calf was still alive at 25 months of age.

3.4. Genetic analysis of the cloned calf

As shown in Table 4, comparison of the microsatellite of alleles in the cloned buffalo calf with the donor cell lines, the donor buffalo and the surrogate mother revealed that the cloned buffalo calf was 100% identity to donor cells and the donor buffalo, which indicated that the cloned buffalo derived from the ear fibroblasts of the nuclear donor buffalo.

4. Discussion

As for cell line cryopreservation, although it results in different kinds of cellular damage such as DNA fragmentation (Men et al., 2003) and ultrastructural cell anomalies (Marfil et al., 2005; Nardid et al., 1997; Taddei et al., 2001), it is still viable for SCNT to produce clones (Lai et al., 2003; Ogura et al., 2000). Lai et al. (2003) recommended that the thawed cells should be cultured further before used for

Table 1
Effects of donor cells on SCNT in buffalo.

Donor cells	Replicates	No. of NT oocytes	No. of couplets fused (%)	No. of embryos cleaved (%)	No. of blastocysts (%)
Freshly thawed ear fibroblasts	20	1382	889 (64.3 ± 5.4) A	456 (51.4 ± 10.6)	84 (9.5 ± 5.4) A
Serum-starved ear fibroblasts	14	693	532 (76.8 ± 5.6) aB	263 (50.7 ± 9.3)	87 (16.2 ± 5.8) B
Cumulus cells	20	883	719 (82.5 ± 9.0) bB	396 (56.9 ± 12.0)	124 (17.9 ± 7.5) B
G1 ear fibroblasts	21	835	566 (77.8 ± 10.4) abB	343 (54.6 ± 12.9)	104 (16.2 ± 4.5) B

Within columns, values with different capital letters ($P < 0.01$) and small letters are different ($P < 0.05$).

Table 2
Efficiency of inter-subspecies and inter-species SCNT in buffalo.

Treatments	Replicates	No. of NT oocytes	No. of couplets fused (%)	No. of embryos cleaved (%)	No. of Blastocysts (%)
Swamp–swamp	11	560	454 (81.3 ± 6.6)	228 (50.9 ± 8.7) A	83 (18.8 ± 8.0) A
Swamp–river	20	883	719 (82.5 ± 9.0)	396 (56.9 ± 12.0) A	124 (17.9 ± 7.5) A
Bovine-buffalo	8	280	230 (81.7 ± 2.2)	184 (77.8 ± 8.5) B	13 (5.9 ± 6.0) B

Within columns, values with different capital letters are different ($P < 0.01$).

Table 3
Embryo transfer record of SCNT blastocysts.

Items	No. of recipients	No. of pregnancies	Conception rate	No. of abortion	Abortion rate	No. of live calves	ET efficiency
Data	13	4	30.8%	3	75.0%	1	7.7%

SCNT, since fresh cultured cells fuse at a higher rate. Cultivation up to the stage of confluence resulted in a high proportion of cells in the G0/G1 phase of the cell cycle due to contact inhibition of proliferation, Zhang et al. (2006) reported that the blastocyst rate of reconstructed embryos from freshly thawed cells was much lower than that from cultured cells in porcine (5.9% vs 17.9%). Coupled with this, serum starvation led to a small but nevertheless significant increase in the proportion of G0/G1 cells (Cho et al., 2005; Poehland et al., 2007; Sun et al., 2008). In this study, all the donor cells adopted had been cryopreserved, and the fusion rate and blastocyst rate of reconstructed embryos from serum-starved ear fibroblasts were significantly higher than those from freshly thawed ones. And one cloned buffalo calf was obtained after transfer of embryos derived from freshly thawed fibroblasts into recipients. The results corresponded to the above reports and demonstrated that even after cryopreservation in a conventional cryoprotectant solution, the ability of somatic cells as donor cells was maintained, and the thawed cells should be cultured before use to promote the SCNT efficiency.

Cumulus cells and ear fibroblasts are most commonly used for SCNT, as they are easy to obtain and result in no injuries to animals. In terms of their effects on SCNT in buffalo, Shah et al. (2009) reported that cumulus cells were better, while Srirattana et al. (2009) reported that cumulus cells were similar to ear fibroblasts. In this study, the cleavage rate and blastocyst rate of embryos derived from cumulus cells and ear fibroblasts were not different from each other. However, the fusion rate of couplets derived from cumulus cells was significantly higher than that from ear fibroblasts. That may be attributed to that the cumulus cells were bigger than fibroblasts after culture, and larger diameter of donor cells was beneficial for fusion. The results demonstrated that cumulus cells and ear fibroblasts had similar potential to support the development of cloned embryos to the blastocyst stage, and both of them could be adopted for SCNT in buffalo.

Serial cloning has practical implications in producing genetically engineered animals. In the current study, the cleavage and blastocyst rates of reconstructed embryos from G1 ear fibroblasts were similar to those of embryos from the G0 ear fibroblasts. Our data were consistent with the observations obtained by Yin et al. (2008) and Kurome et al. (2008), which indicated that the efficiency of second-

Table 4
Microsatellite analysis of buffalos and donor cells.

Loci	Recipient buffalo	Donor buffalo	Donor cells	Cloned buffalo	Random buffalo
ETH10	197, 197	197, 197	197, 197	197, 197	197, 197
TGLA126	104, 104	104, 104	104, 104	104, 104	104, 104
TGLA227	71, 75	75, 77	75, 77	75, 77	75, 77
BM2113	123, 125	123, 125	123, 125	123, 125	123, 125
ETH225	122, 122	122, 122	122, 122	122, 122	122, 122
ETH3	102, 102	102, 102	102, 102	102, 102	102, 102
SPS115	251, 263	245, 251	245, 251	245, 251	254, 257
TGLA153	112, 112	112, 112	112, 112	112, 112	112, 112
BM1862	203, 203	195, 205	195, 205	195, 205	195, 195
BM2934	78, 80	78, 80	78, 80	78, 80	80, 80
BM720	216, 216	216, 216	216, 216	216, 216	216, 216
ILSTS006	276, 276	276, 276	276, 276	276, 276	276, 276

generation cloning was similar to that of first-generation cloning.

Many attempts have been made to explore the feasibility of transferring buffalo somatic cells into bovine oocytes to produce inter-species embryos (Kitiyanant et al., 2001; Lu et al., 2005; Saikhun et al., 2002). All the studies obtained bovine-buffalo blastocysts, while no viable offspring were obtained. In the present study, the developmental competence of bovine-buffalo NT embryos was examined, the cleavage rate of the bovine-buffalo embryos was significantly higher than the swamp–swamp and swamp–river embryos (77.8% vs 50.9%, 56.9%), while the blastocyst rate of inter-species NT embryos was significantly lower than inter-subspecies NT embryos (5.9% vs 18.8%, 17.9%), it was also much lower than those reported by Lu et al. (2005) (3.0–13.3%), Kitiyanant et al. (2001) (17–39%) and Saikhun et al. (2002) (14–34%), this may be due to that the culture conditions for buffalo embryos not being suitable for inter-species embryos, as 64% of cleaved bovine-buffalo embryos developed beyond the 8-cell. The birth of normal *Bos indicus* calves derived from transfer of *Bos indicus* donor cells into *Bos taurus* oocyte recipients demonstrated that inter-subspecies SCNT is a viable approach to produce offspring of closely related subspecies (Meirelles et al., 2001; Steinborn et al., 2002). The results of this study indicate that there was no significant difference in developmental competence between swamp–swamp and swamp–river NT embryos. The birth of a swamp–river cloned buffalo in this study demonstrated that the cytoplasm of swamp buffalo could induce differentiation of somatic cell nuclei derived from river buffalo and support the inter-subspecies NT embryos to develop to full term.

An abortion rate of 75% in this study was similar to that reported by Saikhun et al. (2004). Early loss is frequently associated with functional deficiencies occurring at the onset of placentation as evidenced in sheep and cattle by the lack of placentome development and abnormal vascularization of extraembryonic tissues. These placental abnormalities could lead to severe nutrient deficiencies and induce growth retardation, ultimately causing peri-implantation loss (Sinclair et al., 1999; Wells et al., 1999). It is likely in the inter-species cloned embryos that incomplete reprogramming of the differentiated nucleus was a major constraint to the in vivo developmental potential of these embryos. As the inter-subspecies embryos were transferred to recipients, the immune system of surrogate mothers might exclude the embryos so that placenta could not form normally or formed incompletely after implantation (Jian-Quan et al., 2007). However, further research should be done to explore the precise mechanism of abortion.

In conclusion, this study demonstrates that the developmental competence of swamp–river buffalo NT embryos is not different from that of swamp–swamp embryos, while significantly higher than that of bovine-buffalo embryos. Furthermore, our results suggest that cryopreserved cells should be cultured and serum-starved before use, both the ear fibroblasts and cumulus cells can be adopted for SCNT to produce cloned embryos, and the efficiency of second-generation cloning equals to that of the first-generation cloning in buffalo.

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Influence of ovarian hormones on endocrine activity of gonadotroph cells in the adenohypophysis of lambs during the postnatal transition to prepuberty

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Female sheep

ABSTRACT

There is juvenile hiatus during maturation of larger mammals with relatively long life spans. Using histomorphological and functional criteria we describe the feedback mechanisms which could play a role in the regulation of the gonadotrophic axis during the postnatal transition to the quiescent prepubertal period in sheep. The aim of this study was to determine the influence of ovarian factors on the endocrine activity of gonadotroph cells, the site of synthesis, storage and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in adenohypophyses of weanling and weaned prepubertal lambs. The examination was made in (i) 9-week-old infantiles, suckling lambs undergoing weaning, ovary-intact (OVI) and ovariectomised (OVX) at the 6th week of age, and (ii) 16-week-old juveniles OVI and OVX at the 12th week of age ($n = 5$ per group). Changes in gonadotrophs were assayed with hybridohistochemistry, immunohistochemistry and radioimmunoassay. The percentage of the adenohypophyseal area (PA) occupied by gonadotrophs containing LHβ-mRNA and immunoreactive for LHβ was lower ($P < 0.05$), whereas the PA occupied by cells containing FSHβ-mRNA and immunoreactive for FSHβ was higher ($P < 0.05$) in the 16-week-old OVI lambs in comparison with the 9-week-old ones. The mean concentration and basal level of LH in the peripheral blood plasma were greater ($P < 0.05$) in the 16-week-old OVI lambs in comparison with the 9-week-old group, whereas the circulating FSH was not different. In the OVX 9-week-old lambs, the PA occupied by gonadotrophs containing LHβ-mRNA and the plasma LH concentration, basal level, pulse frequency and amplitude were greater ($P < 0.05$), whereas the PA occupied by cells immunoreactive for LHβ was lower ($P < 0.05$) in comparison with the OVI group. In the OVX 16-week-old lambs, the PA occupied by gonadotrophs containing LHβ-mRNA and immunoreactive for LHβ, the LH plasma concentration, basal level and pulse frequency were ($P < 0.05$) greater in comparison with the OVI group. The PA occupied by gonadotrophs containing FSHβ-mRNA and the plasma FSH concentration were greater ($P < 0.05$) in the OVX 9- and 16-week-old lambs in comparison with the OVI ones. The ovariectomy had no effect on the PA occupied by cells immunoreactive for FSHβ in both age stages. In conclusion, ovarian factors may play inhibitory role in regulating both LH and FSH synthesis rate and release and stimulatory role in regulating LH storage in adenohypophyseal gonadotrophs in infantile lambs. In lambs at the beginning of the juvenile period, ovarian factors may play only inhibitory role in regulating both LH and FSH synthesis and release and LH storage. The effects of ovarian hormones on the gonadotrophin storage, i.e. releasable pools in adenohypophyseal cells, are specific for LH,

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no such effects are apparent on FSH in lambs during the postnatal transition to prepuberty. Thus, the initiation of the juvenile period in female sheep is a function of change of the stimulatory role of ovarian hormones in regulating LH storage to the inhibitory one.

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1. Introduction

The hypophyseal gonadotrophic cells, as the site of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion, are the morphological setting for the endocrine mechanisms that prepare an organism for reproduction. The postinfantile transition to adolescence in mammals involves the neuroendocrine states which evoke significant changes in secretion of hormones (Wańkowska et al., 2006, 2008b; Wańkowska and Polkowska, 2006, 2009). These endocrine changes lead to the full presentation of juvenile phenotype, i.e. physiological readiness to sexual maturation (for review see Veldhuis et al., 2006). The most important feature of juvenile phenotype is an increase in the secretion of LH under changes in the post-translational processing and neuroanatomy of gonadoliberin (GnRH) in the preoptic area-hypothalamus from the infantile to the pubertal pattern (Wańkowska et al., 2008a; Wańkowska and Polkowska, 2009).

The development of the gonadotrophic axis from birth to the juvenile period in female sheep, i.e. during the first 15 weeks of postnatal life, has been poorly described. Information concerning endocrine functions of gonadotroph cells are limited to the patterns of circulating LH and FSH (Foster et al., 1975a,b). These patterns can reflect functional changes of the gonadotrophic axis on the systemic level. Throughout the first 5 weeks of postnatal life, the levels of serum LH are diminished. With the onset of pulsatile LH secretion, beginning 11 weeks after birth, circulating LH increases to levels greater than those observed in adults (Foster et al., 1975b). After the neonatal increase in the concentration of FSH to levels characteristic for adult ewe, the next increase in the levels of serum FSH is observed from 15 to 20 weeks of age (Foster et al., 1975b). In this regard, in newborn female lambs the percentages of LH and LH–FSH cells do not change, whereas FSH cells percentage increases about twofold after birth (Messaoud-Toumi et al., 1993). Fourteen weeks later, i.e. during the initiation of the juvenile period, the gonadotrophic mechanisms involve the different intrahypophyseal regulation of LH and FSH post-transcriptional processing. The development of secretory processes in gonadotrophs during the juvenile period seems to be determined by the decrease in the storage of FSH and the increase in the storage of LH until peripuberty (Wańkowska and Polkowska, 2006). The hypophyseal endocrine mechanisms underlying the changes in gonadotrophins on the systemic level throughout the infantile period, i.e. the transition period between the neonatal and juvenile period, in sheep are yet to be identified and described.

In “precocious species” (e.g. primates), the periods of prenatal and neonatal activation of gonadotrophic axis may be equivalent to the postnatal periods of activation in “altricial species” (e.g. laboratory strains of rodents) i.e. mammals with a short life span and rapid growth and development. In this regard, there is juvenile hiatus during neuroendocrine maturation of larger mammals with relatively long life spans (for review see Ebling, 2005). The reactivation of reproductive development after the quiescent prepubertal period is determined by the reduction in sensitivity of the hypothalamo-hypophyseal unit to inhibitory feedback of the gonadal steroid hormones (Claypool and Foster, 1990; Veldhuis et al., 2006). Thus, it is essential to understand which aspect of the regulation of LH and FSH reflects the feedback action of gonadal hormones before juvenile readiness to sexual maturation in such precocious species as the sheep, inputs from the central nervous system and/or some other aspect? In the present study we focus on the possibility that the patterns of LH and FSH secretion after the neonatal period in female lambs might be related to the influence of the ovarian hormones on changes in releasable pools of gonadotrophin in adenohypophyseal cells.

2. Materials and methods

2.1. Animals, management and experimental design

All procedures were approved by the Local Ethics Committee affiliated to Warsaw Agriculture University (number of opinion 29/2006), according to the Polish Law for the Care and Use of Animals (2 August 1997), and thus were conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

The 3- to 4-year-old ewes of the highly seasonal Polish Longwool breed (features of the breed in Rasali et al., 2006) were mated naturally at a commercial sheep facility (Samoklęski Farm, Poland) and were transported to the Institute of Animal Physiology and Nutrition (Jabłonna, Poland) in September. Sixteen female lambs from twin pregnancy and four from singleton pregnancy were born in February. The animals were fed a diet which provided 100% of the National Research Institute of Animal Production recommendations for pregnancy and lactation in ewes and suckling period in lambs (Norms, 1993). The lambs were penned with dams indoors, in individual pens, under not controlled lighting and temperature conditions present at 52°N latitude and 21°E longitude. Ten lambs were weaned at 65th day of age and were fed ad libitum hay and twice daily a complete pelleted concentrate ration supplemented with vitamins and minerals and containing 17% of protein (norms for fattening period in lambs). This diet assured the optimal tempo of growth.

Four-week-old lambs were divided randomly into four groups ($n = 5$, one from singleton pregnancy and four from twin pregnancy per group) according to different phases of postnatal ontogeny, infancy and the beginning of the juvenile period. Infancy was represented by 9-week-old weanlings (63rd day of age) ovary-intact (OVI) and ovariectomised (OVX) at 39th day of age. They suckled their mothers 3–4 times per 24 h. The juvenile period was represented by 16-week-old lambs (112th day of age) OVI and OVX at 88th day of age. These lambs should begin pubertal ovarian cycles approximately 14 weeks later. The ovariectomy was performed under ketamine/pentobarbitone anaesthesia (8–10 and 10–20 mg/kg body weight, respectively; intravenous injection). The weaned OVI and OVX lambs were maintained in two separate large pens.

A day before collection of blood plasma a jugular venous catheter was inserted and kept with heparinized saline. Blood samples were collected every 10 min for a 5 h (from 08:30 to 13:20 h) in 5- (37th day of age), 9-, 12- (86th day of age) and 16-week-old OVI lambs and 9- and 16-week old OVX lambs. During collection, the lambs were kept in their pens where they could lie down and had unrestrained access to hay, water and/or dams. Blood samples were centrifuged in heparinized tubes and the plasma was stored at −20 °C until LH and FSH assays. At the day of dissection, 9-week-old and 16-week-old OVI and OVX lambs were anesthetized with an intravenous injection of pentobarbitone sodium (20 mg/kg; Biochemie GmbH, Kundl, Austria) and decapitated (from 13:30 to 14:30 h).

2.2. Tissue preparation

The tissue samples for histological assessment were prepared using the clinical standard of formaldehyde fixation with minor modifications and paraffin embedding. Immediately after decapitation, the brains were perfused via the carotid arteries with 0.1% sodium nitrite in 1500 ml 0.1 M phosphate buffered saline (PBS) (Sigma, St. Louis, USA) and then with 1500 ml 0.1 M PBS containing 4% (w/v) paraformaldehyde (Sigma–Aldrich, Seelze, Germany), pH 7.4. Hypophyses were removed from the cranium, postfixed at room temperature for 24 h by immersion in this same fixative, dehydrated in graded alcohol, embedded in paraplast (Sigma, St. Louis, USA) and sliced into 5 μm sections in the sagittal plane.

2.3. Preparation of probes and hybridohistochemistry

Cellular LHβ and FSHβ mRNA was studied by *in situ* hybridisation in combination with immunohistochemical detection of digoxigenin (DIG). Riboprobes were produced using previously obtained homologous sheep double-stranded cDNAs (D'Angelo-Bernard et al., 1990; Pelletier et al., 1992, 1995). The 533 bp oLHβ-cDNA or 900 bp oFSHβ-cDNA were DIG labelled by *in vitro* transcription with SP6 and T7 RNA polymerase using the DIG-labelling kit from Roche Molecular Biochemicals (Meylan, France) and pGEM-TEasy vectors (Promega, Charbonnieres, France). The specificity of probes was confirmed by the absence of a positive signal in sections hybridised with sense probes (data not shown). The non-radioactive *in situ* hybridisation procedure using DIG-labeled cDNA probes was performed according to the Wańkowska et al. (2002). Briefly, slides were fixed with 4% paraformaldehyde in PBS and treated with 0.25% acetic anhydride in 100 mM TEA buffer (triethanolamine-Cl; Sigma, St. Louis, USA). Next, the sections were treated with 200 mM HCl, then with 1% proteinase K (ICN, Aurora, Ohio, USA) dissolved in Tris-EDTA and finally washed in Tris-glycine buffer. Sections were subsequently pre-hybridized and *in situ* hybridisation with DIG-dUTP anti-sense or sense probes diluted 1:100 was carried out at 55 °C in a humid chamber. After incubation, slides were washed in 2× SSC and treated with 20 mg/ml RNase A (Sigma, St. Louis, USA) in 0.5 M NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA. Next, the sections were treated with 30% formamide (Sigma, St. Louis, USA) and washed in 2× SSC. The sections were washed in 100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent (Boehringer, Mannheim, Germany). Next, the sections were incubated with alkaline phosphatase anti-DIG antibody (Boehringer, Mannheim, Germany), diluted 1:500 in the same buffer. Following this, the sections were washed in 100 mM Tris–NaCl (pH 7.5), 150 mM NaCl and in 100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$. Finally, the sections were incubated in buffer containing 75 mg/ml NBT, 50 mg/ml BCIP (Boehringer, Mannheim, Germany) and 10 mM levamisole (Sigma, St. Louis, USA). The colour reaction was stopped in water.

Control sections were incubated with RNase, 1 unit/100 μl 10 mM Tris–HCl buffer, pH 7.9, for 30 min, just prior to the addition of the hybridisation buffer. Controls did not exhibit any staining (data not shown).

2.4. Immunohistochemistry

The LH- and FSH-containing cells in the adenohypophysis were identified by the peroxidase labelled antibody method. Sections were washed in 0.01 M PBS, incubated for 30 min in 2% preimmune normal lamb serum in 0.01 M PBS and 30 min in 0.1% hydrogen peroxide (Chempur, Piekary Śląskie, Poland) in 0.01 M PBS. The sections were incubated with primary rabbit anti-sheep LHβ No. I3 diluted 1:1000 and anti-sheep FSHβ No. P5 diluted 1:200 for 4 days at 4 °C. The antibodies were prepared in C.N.R.S. (Gif sur Yvette, France). Methodological details of their preparation and specificity are described by Hurbain-Kosmath et al. (1990). After incubation with primary antibodies, sections were incubated for 2 h at room temperature with the sheep anti-rabbit Ig [H + L] labelled with peroxidase (BIO-RAD, Steenvoorde, France) diluted 1:40 in 0.1% normal lamb serum in 0.01 M PBS. The colour reaction was developed by incubating sections with 0.05% 3′3-diaminobenzidine tetrahydrochloride chromogen (Sigma, St. Louis, USA) and 0.001% hydrogen peroxide in 0.05 M Tris buffer (ICN, Aurora, Ohio, USA).

For control staining, the primary antiserum was replaced with the same dilution of rabbit serum and anti-hormone serum was inhibited with its homologous antigen. To evaluate the specificity of LH and FSH staining, corresponding antisera were preabsorbed using synthetic rLH32V02 and rFSH32V02 (National Hormone and Pituitary Program, Torrance, CA, USA). Synthetic antigens and antisera were mixed and preincubated for 24 h at 4 °C and then used for the immunohistochemical staining instead of the primary antibodies. Neither of the controls exhibited any specific staining (data not shown).

2.5. *Image analyses*

A type ECLIPSE 80i Nikon projection microscope, Nikon DS-Fi1 camera and NIS-Elements Basic Research image analysis computer system version 2.32 (Nikon Corporation, Tokyo, Japan) were used for histological analyses of the adenohypophysis under objective lens 40×. The cross-sectional area fraction parameter was measured to define the percentage of the total area of the hypophyseal *pars distalis* (PA) that exhibited specific staining. This parameter describes the dimension of the population of cells positively stained for LHβ mRNA, FSHβ mRNA, LHβ or FSHβ. The analyses of the histochemical reaction in gonadotrophs were performed using a mechanical threshold function to select a range of values that were optically identified positive for staining. They were made in four sections of each adenohypophysis, using every 40th section (16 fields of 0.08215 mm^2 measured in each section). Methodological details of the morphometric analyses had been described more precisely in our previous published studies (Wańkowska et al., 2006; Wańkowska and Polkowska, 2006).

2.6. *Determination of LH and FSH in peripheral blood plasma*

The concentration of LH was determined in duplicate 100-μl aliquots by a routine double-antibody radioimmunoassay (RIA) procedure using anti-ovine LH, anti-rabbit-gammaglobulin antisera, and bovine LH standard NIH-LH-B6 (Stupnicki and Madej, 1976). The assay detection limit was 0.312 ng/ml of sample. The intra- and interassay coefficients of variations calculated for control samples at concentrations of 5 and 1 ng/ml of LH were 4 and 10%, respectively.

Plasma FSH concentration was estimated by double-antibody RIA, using anti-ovine-FSH (teri. anti-oFSH) and anti-rabbit-gammaglobulin antisera. The anti-FSH, as well as the FSH standard (teri. oFSH and teri. FSH ig) was kindly supplied by Dr. L.E. Reichert Jr. (Tucker Endocrine Research Institute LLC, Atlanta, Georgia, USA). The assay sensitivity was 1.56 ng/ml and the intra- and inter-assay coefficients of variation were 3.3 ± 1.5 and 11.3 ± 2.2%, respectively.

2.7. *Statistical analyses*

The measurements of area fraction taken for each factor from each section for each adenohypophysis were averaged to obtain a mean estimate for the hypophysis of each animal. Then, the above-mentioned mean data were pooled to represent the individual groups of animals. Thus, the data are reported as the mean of percentage of the total area that exhibited staining according to studied groups ($n = 5$ per group). The effects of age and ovariectomy on the factors studied were assessed by one-way analysis of variance (ANOVA) followed by the post hoc Tukey's test and the Student's *t*-test to determine significant differences between means for individual groups, using the Statistica 6.0 PL software (StatSoft Inc., Tulsa, OK, USA).

The mean concentration of LH or FSH for individual animals was calculated from the area under the curve (the sum of trapezoid areas between the curve and the abscissa). The effects of the age and ovariectomy on plasma hormone concentrations were analysed within groups (between infancy and the beginning of the juvenile period) and between groups (during infancy and the beginning of the juvenile period) by ANOVA followed by the post hoc Tukey's test. Pulse characteristics of LH were calculated using the Pulsar Computer Program developed by Merriam and Wachter (1982) and adapted to operate on an IBM-PC. The cut-off parameters $G_{(n)}$ were set as 5% error rate assuming a normal distribution of data. Analysis was performed individually for every lamb and included the entire sampling period. The significance of differences in LH pulse frequency (defined as the number of identified pulses per collecting period) within groups was assayed by the Wilcoxon test and between groups by the Mann–Whitney test. The levels of significance for differences between data of amplitude and peak length of LH pulses within and between groups were calculated by the nonparametric ANOVA rank Kruskal–Wallis test.

All data are expressed as means ± SEM. Significance was defined the $P < 0.05$.

3. Results

3.1. *Histochemical detection of LHβ mRNA, FSHβ mRNA, LH and FSH*

The optically detected population of LHβ mRNA-positive cells was greater in 9-week-old than in 16-week-old OVI lambs (Fig. 1), whereas the population of FSHβ mRNA-positive cells was optically greater in 16-week-old than in 9-week-old OVI lambs (Fig. 1). Populations of LHβ mRNA- and FSHβ mRNA-positive cells were optically greater in OVX lambs than in OVI ones from both age stages (Fig. 1). These qualitative results were reflected by the higher ($P < 0.05$) PA occupied by gonadotrophs containing *in situ* hybridised mRNAs (Fig. 2A and B).

The optically detected population of gonadotrophs immunoreactive for LH and the PA occupied by these cells were greater in 9-week-old than in 16-week-old OVI lambs and in 9-week-old OVI lambs compared to OVX ones ($P < 0.05$; Figs. 1 and 2C). At the 16-week-old stage, an inverse situation was observed in OVI lambs versus OVX lambs (Fig. 2C). In the case of cells immunoreactive for FSH, their population was optically greater in 16-week-old than in 9-week-old OVI lambs (Fig. 1), what was reflected by the higher ($P < 0.05$) PA occupied by gonadotrophs containing FSH (Fig. 2D). The population of FSHβ-immunoreactive gonadotrophs was similar in adenohypophyses of OVI and OVX lambs from the 9-week-old (Fig. 1) and 16-week-old stage, what was reflected by the similar PA occupied by gonadotrophs containing FSH (Fig. 2D).

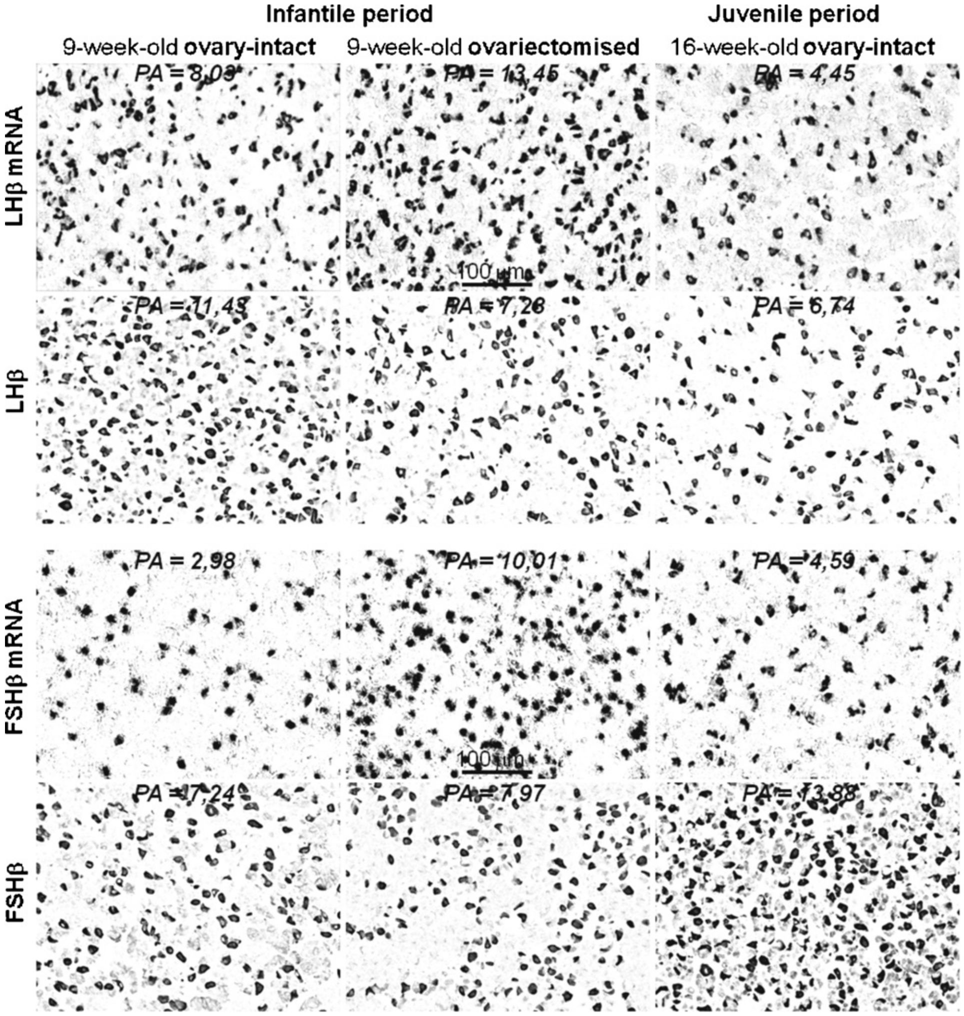

Fig. 1. Gonadotroph cells containing *in situ* hybridised mRNA transcripts encoding LHβ- and FSHβ-subunit or immunoreactive for LHβ and FSHβ subunits in the adenohypophyseal *pars distalis* of the representative ovary-intact (OVI) versus ovariectomised (OVX) infantile lamb and juvenile OVI lamb. PA, the percentage of area occupied by cross-sections of stained cells. Note a great population of gonadotrophs containing LHβ mRNA and FHSβ mRNA and a small population of LH-containing gonadotrophs in the OVX infantile lamb compared with the OVI one. In the juvenile lamb note a great population of FSH-containing gonadotrophs.

3.2. LH and FSH in peripheral blood plasma

The plasma LH concentration and basal level was lower in 5- and 9-week-old OVI lambs in comparison with 12- and 16-week-old ones ($P < 0.05$; Fig. 3). The frequency of LH pulses increased from 5 until 12 weeks of age ($P < 0.01$) and decreased at 16 weeks of age ($P < 0.05$). The LH pulse amplitude was lower in 5-week-old OVI lambs in comparison with the later age stages ($P < 0.05$) and the peak length was lowest in 5-week-old OVI lambs and highest in 16-week-old ones ($P < 0.01$; Fig. 3). The plasma FSH concentration was higher in 16-week-old OVI lambs in comparison with the previous age stages ($P < 0.05$; Fig. 4).

In OVX 9- and 16-week-old lambs the LH plasma concentration, basal level and frequency of LH pulses ($P < 0.05$; Fig. 3) and plasma concentration of FSH ($P < 0.01$; Fig. 4) were greater in comparison with the OVI groups. The LH pulse amplitude was higher only in OVX 9-week-old lambs in comparison with the OVI group ($P < 0.05$; Fig. 3). The peak length was lower in OVX 16-week-old lambs than in OVI ones ($P < 0.05$; Fig. 3). In OVX lambs the mean concentration of FSH was huge in comparison with the mean concentration of LH in both age stages (84.9 ± 10.06 versus 14.0 ± 0.80 ng/ml in 9-week-old group and 42.5 ± 3.46 versus 11.7 ± 0.92 ng/ml in 16-week-old group; $P < 0.001$; Figs. 3 and 4). The plasma concentration of both LH and FSH was higher in 9-week-old OVX lambs than in 16-week-old ones ($P < 0.05$; Figs. 3 and 4).

4. Discussion

The study elucidates the mechanisms of ovarian hormones feedback at a cellular level in adenohypophysis during the transition from the neonatal to juvenile period in sheep. The converse links demonstrated between presence of ovaries and dimension of population of LH-containing gonadotrophs in studied stages provide evidence that ovarian factors may play a role in regulating LH storage during

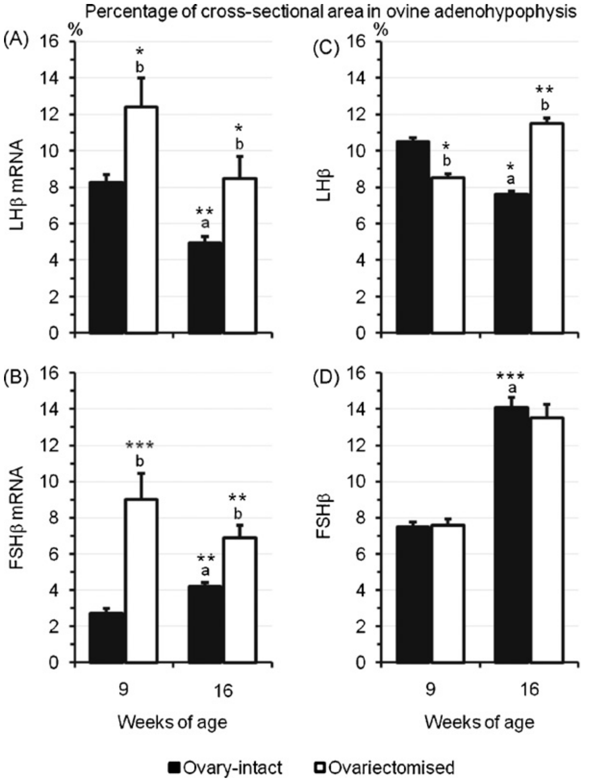

Fig. 2. The percentage of adenohypophyseal area (cross-sectional area fraction) occupied by the gonadotrophs containing *in situ* hybridised LHβ-mRNA (A) or FSHβ-mRNA (B) and immunoreactive for the LHβ (C) or FSHβ (D) subunit within the hypophyseal pars distalis of infantile (9-week-old) and juvenile (16-week-old) lambs ovary-intact (OVI) and ovariectomised (OVX) 4 weeks before the hypophyses dissection. Values are means (for every $n = 5$) ±SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; a and b: a statistical significant difference for OVI and OVX versus OVI lambs, respectively.

infancy and at the beginning of the juvenile period. The low accumulation of LH in absence of ovaries in infantiles suggests the stimulatory role, whereas the high storage of LH after ovariectomy in juvenile lambs suggests the inhibitory role. In contrast to LH, the ovariectomy had no effect on the accumulation of FSH in gonadotrophs in both age stages during the postnatal transition to prepuberty. However, the increase in the dimension of subpopulation of FSH-containing gonadotrophs was accompanied by the decrease in the dimension of subpopulation of LH-containing cells at the beginning of the juvenile period. As in the case of lambs undergoing the transition to the juvenile period after the period of weaning, in newborn female lambs the percentage of FSH-containing gonadotrophs increases after birth, whereas LH-containing cells percentage do not change (Messaoud-Toumi et al., 1993). These findings suggest that in precocious species the secretion of both LH and FSH from birth to prepuberty should be differentially regulated by neonatal and postweaning changes in the proportion of gonadotroph subtypes within the total adenohypophyseal gonadotroph population, as was found previously for prepubertal and pubertal lambs (Wańkowska and Polkowska, 2006; Wańkowska et al., 2008a) and rhesus monkey (Meeran et al., 2003). In contrast to precocious species, in altricial species such as the rat, the numbers of gonadotrophs per hypophyseal tissue and the amounts of both LH and FSH in hypophyseal cells increase during infancy until the juvenile period. Then the number of LH cells remains high until puberty (Jansen, 1982). Altogether, the heterogeneity in the dimensions of gonadotroph subpopulations containing LH and FSH in the postnatal transition to prepuberty is related to some adjustment of the ovarian hormones feedback loop, i.e. the change of the positive feedback effect on the number of gonadotrophs containing LH to the negative one. Such homeostatic feedback mechanism seems to be specific for the regulation of the gonadotrophic axis during the beginning of the quiescent prepubertal period in the female sheep, which is a representative of larger mammals with longer life span.

These effects of the age and ovariectomy on the gonadotrophs containing LH and FSH were accompanied by the different effects on the accumulation of mRNA for β subunits of LH and FSH. A feature of the transition from the infantile to juvenile period is the decrease in the dimension of gonadotroph population containing LHβ mRNA and increase in the case of cells containing FSHβ mRNA. However, the LHβ and FSHβ mRNA abundance in absence of ovaries in both infancy and at the beginning of the juvenile period suggests the inhibitory role of ovarian hormones in regulating the transcription rate of both LHβ and FSHβ. The lamb and rat differ in their LH synthesis rate during postnatal development until the juvenile period. In female rats, LHβ and FSHβ mRNA contents rise throughout infancy to the juvenile period (Zapatero-Caballero et al., 2004). However it should be noted that in both the weanling lamb and infantile rat FSHβ mRNA is dramatically regulated in the adenohypophysis. In rats the source of this regulation does not appear to be GnRH (Wilson and Handa, 1997) but probably gonadal steroid hormones (Wilson and Handa, 1998). The previous studies on adult ewes (Baratta et al., 2001; Clarke, 2002; DiGregorio and Nett, 1995; for review see Nett et al., 2002) and infantile female rats (Wilson and Handa, 1998) indicate that gonadotrophs are capable of responding directly to gonadal hormones, and thus may play a role in the selective regulation of LH and FSH expression and secretion *in vivo*. It was previously suggested, that the negative feedback of estradiol on LH and FSH secretion mainly targets the bihormonal gonadotroph cells and occurs, at least in part, directly at the hypophysis level in adult ewes (Molter-Gerard et al., 2000). Altogether, the discordant changes in the transcription rate are similar to the changes in accumulation of LH and FSH in the ovine adenohypophyseal endocrine cells during the postnatal transition to prepuberty. The decrease in the accumulation of LHβ mRNA and increase in the accumulation of FSHβ mRNA after the infantile period are under negative feedback effects of gonadal hormones in female lambs.

The patterns of circulating hormones image the described changes in the gonadotrophin transcription rate. Circulating LH was uniformly low in infantile stages and then uniformly high beyond the period of weaning. It was previously suggested that low tonic LH secretion, presumably in the form of slow pulses, is necessary for development or maintenance of ovarian function before puberty

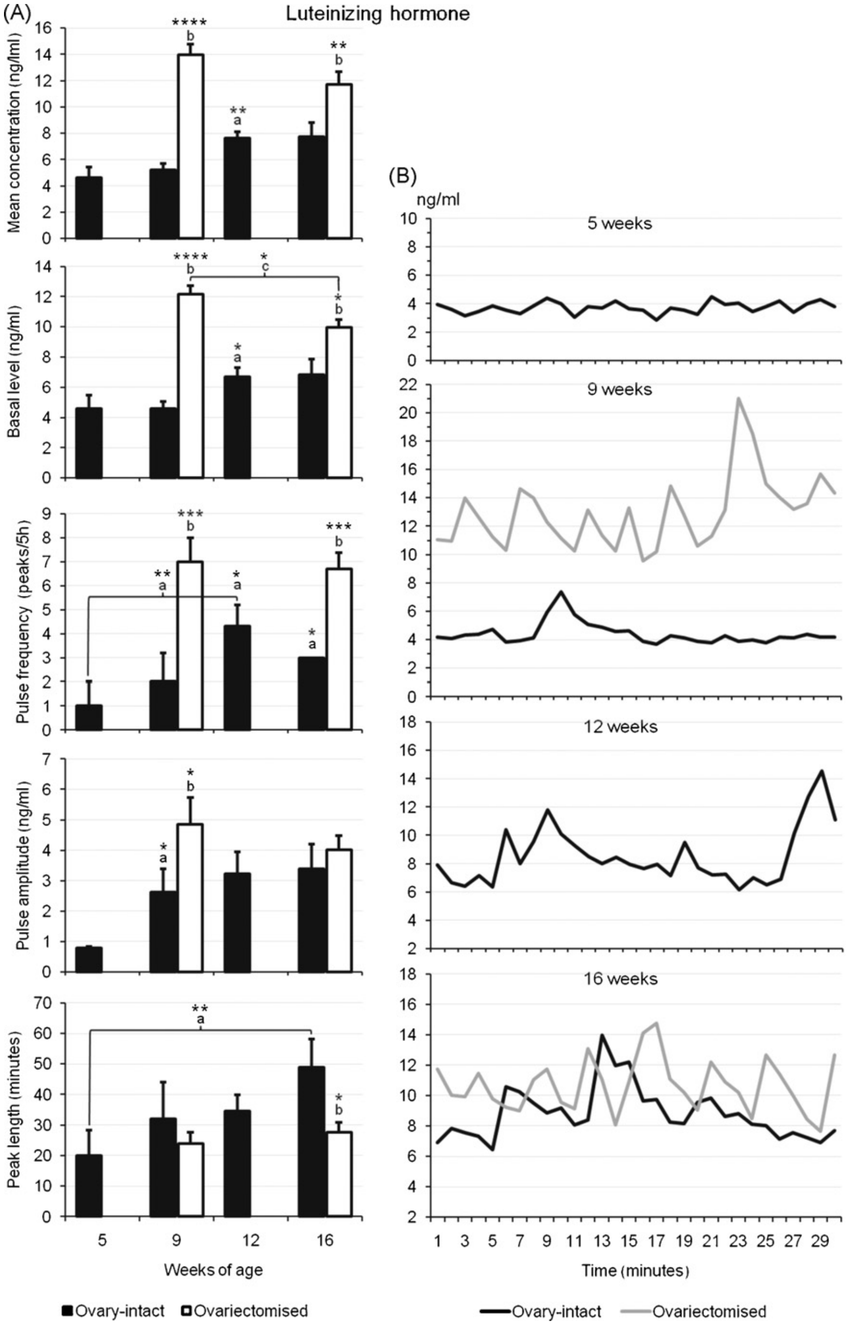

Fig. 3. (A) The peripheral blood plasma luteinizing hormone (LH) mean concentration, basal level, pulse frequency, pulse amplitude, peak length in weanling (5- and 9-week-old) and weaned prepubertal (12- and 16-week-old) lambs ovary-intact (OVI) and ovariectomised (OVX) 4 weeks before blood collection. Values are means (for every $n = 5$) ±SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; a–c: a statistical significant difference for OVI, OVX versus OVI and OVX versus OVX lambs, respectively. (B) The pulsatile pattern of LH release in individual lambs.

in lambs (Foster et al., 1986). The FSH plasma levels were uniform until the 11th week of age and there was prepubertal increase in the circulating FSH in 16-week-old lambs. The stages of the developmental increase in serum LH and FSH levels correspond with the stage of the juvenile period beginning after the weaning period in our experimental model. However, the release of both gonadotrophins during the infantile–juvenile transition period seems to be under negative feedback effects of gonadal hormones. The changes in the patterns of both gonadotrophins release are similar in lambs and infantile rats, as is in the case of the synthesis rate. The plasma LH and FSH rose to higher levels during infantile development until the beginning of the juvenile period and estradiol likely is the major candidate in stimulation of FSH secretion in the infantile female rat (Herath et al., 2001). In this regard, estradiol is capable of

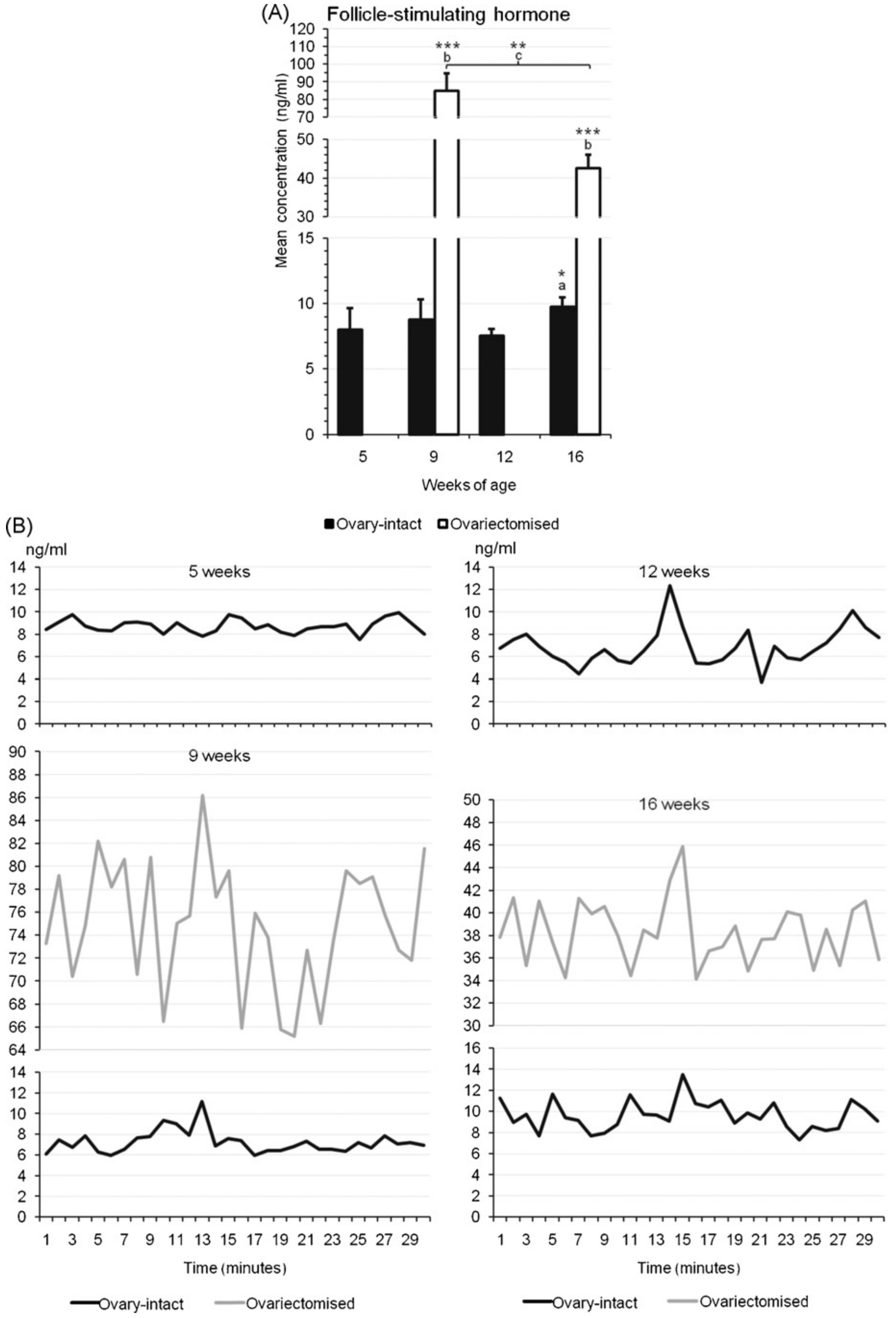

Fig. 4. (A) The mean concentration of peripheral blood plasma follicle-stimulating hormone (FSH) in weanling (5- and 9-week-old) and weaned prepubertal (12- and 16-week-old) lambs ovary-intact (OVI) and ovariectomised (OVX) 4 weeks before blood collection. Values are means (for every $n = 5$) ± SEM; $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$; a–c: a statistical significant difference for OVI, OVX versus OVI and OVX versus OVX lambs, respectively. (B) The pattern of FSH release in individual lambs.

altering secretion of FSH and LH in the absence of GnRH in adenohypophyseal cells from anestrous ewes (Baratta et al., 2001). However, an explanation of the dramatic increase in FSH levels after ovariectomy, as compare with more moderate increase in LH in the current study, could lie in the paracrine or endocrine influences of factors that may selectively affect the secretion of only FSH. It is conceivable that ovariectomy suppressed inhibin, the protein produced by developing ovary follicles and affecting FSH in gonadotroph cells (Burger et al., 2001; Farnworth, 1995; Gregg et al., 1991). It should be noted, that in the female rat the inhibin regulation of hypophyseal FSH secretion, through its negative feedback, begins to operate during the transition from the infantile to the juvenile prepubertal period (Herath et al., 2001). Altogether, the endocrine transition from the neonatal to juvenile period in female sheep is related to similar regulation on the level of LH and FSH synthesis and release and different regulation on the level of their storage. The increase in the LH and FSH synthesis and release rate after the neonatal period is under negative feedback effects of gonadal hormones in female lambs.

In lambs during the postnatal transition to prepuberty, the rate of FSH release is related to the rate of synthesis, i.e. it is constitutive. The release of FSH in mammals is primarily constitutive (Farnworth et al., 1988, 1995). It is trafficked to the site of exocytosis shortly after synthesis, and released (Crawford and McNeilly, 2002). The direct relationship between the level of hypophyseal FSHβ mRNA and plasma FSH is representative for constitutively secreted hormones (Brooks et al., 1992; Crawford and McNeilly, 2002; McNeilly, 1988). In contrast to FSH, the secretory pattern of LH does not reflect its transcriptional rate but rather the translational processing in female lambs which begin the juvenile period. This postinfantile secretory pattern does not reflect LH post-translational intrahypophyseal mechanisms due to the storage of secretory granules within the gonadotrophs, as is in the case of older prepubertal and pubertal lambs (Wańkowska and Polkowska, 2006; Wańkowska et al., 2008a) and in ewes during the oestrous cycle (Crawford et al., 2000; Crawford and McNeilly, 2002). The cellular mechanism observed in weanlings in contrast to juvenile lambs is similar to the positive feedback action of oestrogen which mobilizes LH-containing, but not FSH-containing secretory granules in gonadotrophs of cycling ewes (Currie and McNeilly, 1995; Thomas and Clarke, 1997). It should be remained, that for the operation of a regulatory/storage pathway for the secretion of LH is required GnRH; no such mechanism appears for FSH (McNeilly et al., 1991). Altogether, whilst a pivotal feature of the GnRH/LH secretory pathway in the ovine quiescent prepubertal period is ability of LH protein to be stored (Wańkowska and Polkowska, 2006, 2009; Wańkowska et al., 2008a), an important feature of the GnRH/LH axis during postnatal transition to prepuberty is the ability of LH to be synthesized and released.

5. Conclusions

The increase in both the LH and FSH synthesis rate and release is actively inhibited, whereas increase in the storage of FSH in adenohypophyseal cells is passive under ovarian feedback effects during the transition from the infantile to juvenile period in lambs. An endocrine feature of gonadotroph cells during initiation of the juvenile period is decrease in accumulation of LH. From a cellular perspective of gonadal–hypophyseal feedback loop, the beginning of the juvenile period is a function of change of the stimulatory role of ovarian hormones in regulating LH storage, i.e. releasable pools of LH in adenohypophyseal cells, to the inhibitory one. Such homeostatic feedback mechanisms seem play an important role in the regulation of the gonadotrophic axis during the postnatal transition to the quiescent prepubertal period in sheep.

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The negative effects of a short period of maternal undernutrition at conception on the glucose–insulin system of offspring in sheep

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ABSTRACT

Maternal undernutrition during pregnancy has negative effects on fetal development and offspring health. However, the effect of maternal undernutrition about the time of conception on neonatal outcome is not clear. We investigated the impact of ewe undernutrition during the periconceptional period on offspring body weight and cortisol and insulin concentrations at birth and the insulin response to a glucose challenge and the cortisol response to a corticotrophin releasing hormone (CRH) challenge at 10 weeks of age. Ewes (76 ± 1 kg) were fed 70% (restricted) or 110% (control) maintenance requirements from 28 days prior until 7 days after mating and characteristics of their lambs were assessed. Restricted ewes lost 2.6 ± 0.3 kg ($n = 35$) over the treatment period compared to control ewes which gained 1.7 ± 0.3 kg ($n = 31$) ($P < 0.01$). Male lambs born to ewes that were nutritionally restricted had significantly lower plasma glucose concentrations at birth and a higher insulin response to the glucose challenge at 10 weeks; female offspring were not affected. Lamb weight and cortisol response to CRH at 10 weeks was unaffected by treatment. We conclude that a short period of maternal undernutrition about the time of conception did not affect the adrenal function of offspring but that there was a significant negative effect on the glucose–insulin system of male but not female offspring.

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1. Introduction

The early origins of adult disease hypothesis state that environmental influences acting in fetal life are reflected in impaired growth and development, which permanently affects structure and metabolism, leading to increased risk of metabolic disease in later life (Barker, 2007). A world wide series of epidemiological and experimental studies has developed this hypothesis further to include associations with increased risks of obesity, type II diabetes, insulin resistance, impaired glucose tolerance, hypertension and cardiovascular disease in later life (McMillen and Robinson, 2005). In the face of altered nutrient supply during a sensitive period, the fetus or embryo acts by programming its organs and tissues resulting in long-term consequences for function (Lucas, 1991).

To date, many of the studies in humans have arisen from epidemiological cases so that, whilst they identify phenomena, they do not give an insight into the underlying mechanisms involved (Hoet and Hanson, 1999). Rodent models offer many advantages such as short gestation length and transgenerational studies (McMillen et al., 2008); however, there are many disadvantages including the fact that they are litter bearing species with short gestations and that they fail to develop the same disease profile as humans. The sheep model is more akin

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to human beings in that sheep are precocial species that have a long gestation and generally produce one to two offspring per pregnancy. After a period of organogenesis in early gestation the sheep fetus, like the human, has the ability to create a range of appropriate metabolic and physiological responses to changes in the uterine environment to ensure fetal survival, but which in turn may result in adverse health outcomes in later life (McMillen et al., 2008).

There are a number of reports in the literature where ruminants have been used as a model for studying the effects of altered maternal nutrition during gestation on fetal development and offspring health and development (Duffield et al., 2009; Sullivan et al., 2009, 2010; Wallace et al., 2010). In agreement with the early origins of adult disease hypothesis (above), there are wide ranging consequences for different types of maternal diet (e.g. protein and energy content, timing of treatment, age and growth status of the dam) during gestation on offspring health but there are very few studies that have critically examined the outcome of the effects when imposed during the oocyte and embryo stages (before organogenesis) of development. Recent research in sheep has drawn attention to the ways in which the environment of the developing follicle, oocyte and early embryo or fetus in the first trimester of pregnancy can induce phenotypic changes (Hanson and Gluckman, 2005). The findings of a number of studies indicate that lambs born to ewes who were undernourished by 15–50% between 1 and 70 days of gestation, had an altered hypothalamo-pituitary-adrenal (HPA) axis in late gestation, as well as increased HPA activity in early postnatal life (Hawkins et al., 2000) and an increase in body weight and fat deposition and dysregulated glucose uptake in adolescence (Chadio et al., 2007). Maternal undernutrition imposed during the periconceptional period (for up to 60 days before and 70 days after conception) also altered HPA function during later fetal (Hawkins et al., 1999, 2001; Edwards et al., 2002; Edwards and McMillen, 2002; Bloomfield et al., 2003b) and early postnatal life (Hawkins et al., 2000) and post-pubertal life in sheep (Todd et al., 2008). Studies of late gestation fetuses in ewes that were undernourished by 30–50% between 60 days before and 7–30 days after mating showed reduced growth rate, higher insulin and higher glucose (Oliver et al., 2001, 2005) and cortisol concentrations (Bloomfield et al., 2004).

Most studies of undernutrition during pregnancy in sheep have only extended observations on the offspring as far as late gestation fetal development and therefore there is limited information on the longitudinal effects of maternal undernutrition during pregnancy on postnatal offspring health and development. Studies that have looked at the effects of periconceptional undernutrition on offspring health and development have focused on a large window of maternal undernutrition that extends into early to mid-pregnancy. The aim of this study was to investigate the impact of maternal periconceptional undernutrition (28 days prior to 7 days after conception, i.e. a short window), during oocyte and early embryo development, on the function of the glucose–insulin system and the HPA axis at birth and 10 weeks of age.

2. Materials and methods

2.1. Animals and nutritional management

This experiment was approved by the UCD Animal Research Ethics Committee. All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1897) and European Community Directive 86/609/EC.

Sixty-eight Suffolk-cross multiparous ewes ranging in age from 2 to 5 years were randomly selected from a larger flock of ewes. All ewes were accommodated in individual pens on slatted floors from 35 days before until 7 days after conception (conception was early in October). All ewes underwent an acclimatization period of 1 week prior to being assigned to treatments (on Day −28) in order to adapt to the new housing environment and diet. Twenty-eight days before mating ewes were blocked on weight and age and randomly assigned to one of two treatments: control ($n = 33$), receiving a 1.1× maintenance diet (to maintain a constant body weight) or restricted ($n = 35$), receiving a 0.7× maintenance diet. The metabolisable energy (ME) requirements for maintenance were estimated as 0.42 MJ ME/kg $W^{0.75}$ (Robinson, 1981). Ewe bodyweight (BW) was recorded weekly and a weighed quantity of diet was offered accordingly. The diet was offered then on two occasions each day (at 0900 and 1600). The diet consisted of barley, molassed beet pulp, grass meal, vitamins and minerals; 100 g of wheat straw was offered to each ewe once per day (at 1200). Ewes were exposed to natural photoperiod and received ad libitum fresh water. On Day −13, an intravaginal progestagen sponge was administered to each ewe and removed on Day −1 for synchronization of oestrus. On Day 0 (oestrus), ewes were temporarily moved into a large shed and exposed to fertile rams for breeding at a ratio of 7 ewes/ram. Rams were randomized across the dietary groups and removed after 20 h. Ewes were returned to their individual pens. On Day 7 of gestation ewes were returned to the flock and were maintained under standard commercial husbandry practices for pregnant ewes (at pasture until Day 82 of gestation then housed and offered grass silage ad libitum supplemented with 500–700 g concentrate meal).

Pregnancy and litter size were determined in ewes on Day 75 of gestation using transabdominal ultrasonography. Only data from ewes giving birth to single ($n = 31$) or twin ($n = 35$) lambs are reported here (two ewes had triplets). After birth, lambs remained with their mothers until weaning at 14 weeks of age. Throughout the experiment the lambs were kept at pasture under standard husbandry conditions.

2.2. Lamb characteristics

Within minutes of parturition, lamb birth weight was recorded and blood samples from newborn lambs were collected before their first feed for the measurement of plasma glucose, insulin and cortisol. The placenta was also weighed (total fetal membranes) and in the case of twins, individual placentae were not separated and the total weight of all the membranes was recorded.

2.3. Glucose tolerance test and corticotrophin releasing hormone (CRH) challenge

At 10 weeks of age, lambs were subjected to a glucose tolerance test and a CRH challenge. The lambs were given a 24 h acclimatization period to housing conditions (animals grouped in pens) and had a fasting period of 14 h (their mothers were removed, no feed was provided, water was freely available) prior to the challenge. Glucose (500 mg of glucose/kg BW as a 50% glucose–saline (w/v) solution) was administered as a bolus infusion (about 10 s) via a 16-gauge jugular vein catheter (Quick-Cath, Acravet Ltd., Co. Cork). Blood samples (8 ml divided equally between heparinised vacutainer for insulin and a sodium oxalate vacutainer for glucose) were collected at −10, 0, 10, 20, 60, and 120 min relative to glucose administration (Kitchalong et al., 1995). During blood collection lambs were kept in the group pen and were gently manually restrained as needed.

CRH (bolus of 0.5 μg CRH/kg BW; Sigma; reconstituted in a 5 μg/ml saline solution) (Bloomfield et al., 2003a) was given via a 19 gauge needle 2.5 h after glucose administration (described above). Blood samples (4 ml collected into a heparinised vacutainer) were collected at −90, −20, 10, 30, 60, 120, 180 and 240 min from CRH administration. All blood was centrifuged at 1600 × *g* for 20 min at 4 °C. The plasma was separated and stored at −20 °C until analysis.

2.4. Hormone assays

Plasma cortisol concentrations were determined using a commercially available RIA kit (Corti-cote; MPbio, UK) adapted and validated for bovine plasma (Fisher et al., 1996). The mean intra-assay and inter-assay coefficients of variation (CV) for samples containing low (5.47 ± 0.48 ng/ml), medium (16.12 ± 0.69 ng/ml) and high (76.92 ± 3.74 ng/ml) cortisol concentrations were 5.7%, 4.8% and 4.6%; 5.6%, 4.6% and 4.6%, respectively. The sensitivity of the assay was 0.07 ng/ml.

Plasma insulin concentrations were determined using a two-site fluoroimmunometric assay (AutoDELFIA insulin, Perkin Elmer Life Sciences, Wallac Oy, Turku, Finland; catalogue no. B080-101) and validated for bovine plasma (Ting et al., 2003). Samples with insulin concentrations that were higher than the highest standard were diluted in buffer until they were on the curve. The mean intra-assay and inter-assay CV for samples containing low (2.09 ± 0.49 μU/ml), medium (4.19 ± 1.00 μU/ml) and high (11.00 ± 2.41 μU/ml) insulin concentrations were 4.6%, 3.2% and 2.6%; 4.5%, 3.3%, 2.8%, respectively. The sensitivity of the assay was 0.5 μU/ml.

Plasma glucose concentrations were determined by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase enzymes to measure the formation of NADH photometrically (Randox imola system, Randox Laboratories Ltd., Co. Antrim, UK). The mean inter-assay CV for samples containing low (6.23 ± 0.04 mM/l) and high (16.23 ± 0.07 mM/l) cortisol concentrations were 2.21% and 1.61%, respectively. The sensitivity of the assay was 0.64 mM/l.

2.5. Statistical analysis

All data were checked for adherence to a normal distribution. Log transformations of the data were carried out where appropriate. Data on ewe characteristics were initially analysed using two-way ANOVA including the effects of treatment, birth rank (single versus twin) and the interaction between treatment and birth rank in the model. Where no effects of birth rank or interactions were present, these effects were eliminated from the final model and data were further analysed for treatment effects only. Lamb birthweight, placental weight, body dimensions and blood metabolites at birth were analysed using two-way ANOVA including the effects of treatment, birth rank and the interaction between treatment and birth rank in the model. Plasma glucose, insulin and cortisol concentrations over time were analysed by repeated measures ANOVA. All statistical analyses were conducted using SAS (Version 8. Cary, USA: SAS Institute Inc.; 2000). Unless otherwise stated, results are presented as the untransformed means ± SEM. Post hoc analysis was performed with a Tukey's HSD test as needed. Data for male and female offspring were analysed and are presented separately.

3. Results

3.1. Characteristics of ewes

The initial weights of the ewes assigned to the two treatments were not different (control: 75.66 ± 1.12 kg, $n = 33$; restricted: 76.00 ± 1.01 kg, $n = 35$). The change in maternal live weight from 21 days before to 7 days after conception was affected by treatment ($P < 0.001$) with the restricted group losing weight (−2.60 ± 0.27 kg, $n = 35$) and the control group gaining weight (1.67 ± 0.26 kg, $n = 33$). Gestation length was similar ($P > 0.05$) in both groups (control, 147.8 ± 0.4; restricted 147.2 ± 0.3 days).

3.2. Characteristics of lambs at birth

Lamb birth weight was unaffected by treatment; however, the birth weight of twins was lower than singles for both males ($P < 0.001$) and females ($P < 0.010$) (Table 1).

Plasma insulin and cortisol concentrations at birth were not significantly different between the treatment groups. The only effect of treatment at birth was on plasma glucose concentrations in male lambs that were lower ($P < 0.05$) in the restricted single animals compared to the control single animals (Table 1). Twins had lower plasma glucose (males, $P = 0.005$; females, $P = 0.009$) and insulin (females, $P = 0.016$) concentrations at birth compared to single lambs (Table 1).

3.3. Response to glucose and CRH challenges at 10 weeks of age

The response of plasma glucose concentrations to the administration of the glucose bolus was not different between the groups for both male ($P = 0.8166$) and female

Table 1
Characteristics at birth of single and twin male and female lambs born to ewes fed a controlled diet (C = 1.1× maintenance) or a restricted diet (R = 0.7× maintenance) from 28 days before to 7 days after conception.

Variables	Control	Restricted	Probability		
			C versus R	Single versus Twin	Interactions
Female lambs					
Number					
Single	7	7			
Twin	16	18			
Weight at birth (kg)					
Single	5.7 ± 0.3	6.2 ± 0.3	NS	0.0016	NS
Twin	5.2 ± 0.1	4.9 ± 0.3			
Placental weight (kg)					
Single	0.56 ± 0.06	0.33 ± 0.10	NS	0.010	NS
Twin	0.65 ± 0.05	0.71 ± 0.07			
Glucose (mM/l)					
Single	3.6 ± 0.6	3.6 ± 0.5	NS	0.0095	NS
Twin	2.4 ± 0.2	2.6 ± 0.3			
Cortisol (ng/ml)					
Single	16.1 ± 2.9	17.8 ± 3.9	NS	NS	NS
Twin	16.9 ± 1.9	15.1 ± 1.2			
Insulin (ng/ml)					
Single	4.92 ± 1.02	3.38 ± 0.62	NS	0.0160	NS
Twin	1.91 ± 0.37	2.85 ± 0.92			
Male lambs					
Number					
Single	7	10			
Twin	20	16			
Weight at birth (kg)					
Single	6.8 ± 0.3	6.4 ± 0.2	NS	<0.0001	NS
Twin	5.2 ± 0.2	5.6 ± 0.2			
Placental Weight (kg)					
Single	0.59 ± 0.07	0.53 ± 0.06	NS	0.0173	NS
Twin	0.78 ± 0.12	0.87 ± 0.06			
Glucose (mM/l)					
Single	5.9 ± 1.0	3.3 ± 0.5	0.0240	0.0052	0.0067
Twin	3.0 ± 0.2	3.3 ± 0.3			
Cortisol (ng/ml)					
Single	19.8 ± 3.1	19.0 ± 2.0	NS	NS	NS
Twin	16.8 ± 1.1	18.1 ± 2.0			
Insulin (μU/ml)					
Single	2.68 ± 0.93	4.34 ± 1.97	NS	NS	NS
Twin	2.21 ± 0.47	3.69 ± 0.88			

NS $P > 0.05$.

lambs ($P = 0.675$), but changed significantly over time (<0.001) (Figs. 1 and 2). In response to the glucose challenge, insulin concentrations also changed significantly over time ($P < 0.001$), but were not affected by birth rank (single versus twin) (males, $P = 0.558$; females, $P = 0.163$). However, in response to the glucose challenge, insulin concentrations were affected by maternal diet in male offspring ($P = 0.047$); lambs born to nutritionally restricted mothers had a greater insulin response than lambs born to control ewes (Fig. 3). No interaction terms were significant in the male offspring. The effect of maternal diet (treatment) on insulin response to glucose in female offspring was not significant ($P = 0.589$; Fig. 4); however, there was a treatment-by-time interaction with the female restricted group returning to baseline concentrations at a faster rate than the control group ($P = 0.0375$) resulting in lower insulin concentrations 120 min after the challenge in the restricted compared to control animals (Fig. 4).

Plasma cortisol concentrations changed over time ($P < 0.001$) in response to the CRH challenge for both male and female offspring but were unaffected by treatment (male, $P = 0.157$; female, $P = 0.955$) or birth rank for male offspring ($P = 0.280$) when the whole profile was considered (Figs. 5 and 6). However, there was a non-significant trend for the peak concentration of cortisol reached after CRH administration to be lower ($P = 0.094$) in the female lambs born to nutritionally restricted ewes compared to control animals (single control 50.0 ± 7.2; single restricted 42.47 ± 1.9; twin control 44.4 ± 4.0; twin restricted 37.1 ± 3.3 ng/ml). Birth rank in female offspring affected the response to CRH, with the twin group having a significantly lower concentrations of cortisol in response

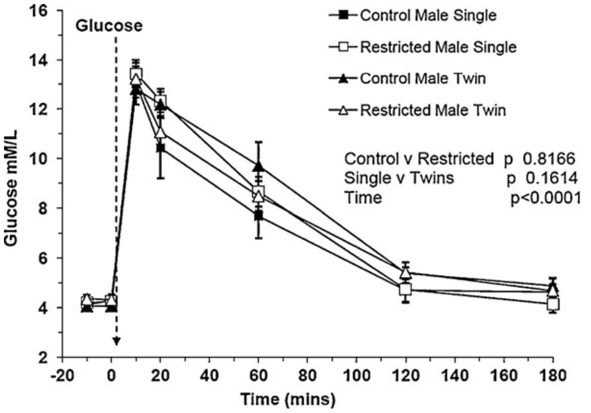

Fig. 1. Plasma glucose concentrations in male lambs at 10 weeks of age plotted relative to an intravenous infusion of glucose (0.5 g/kg body weight in saline; 50%, w/v). Single (square symbols) or twin (triangle symbols) male lambs were born to ewes fed a control diet (1.1× maintenance; closed symbols) or a restricted diet (0.7× maintenance; open symbols) from 28 days before to 7 days after conception.

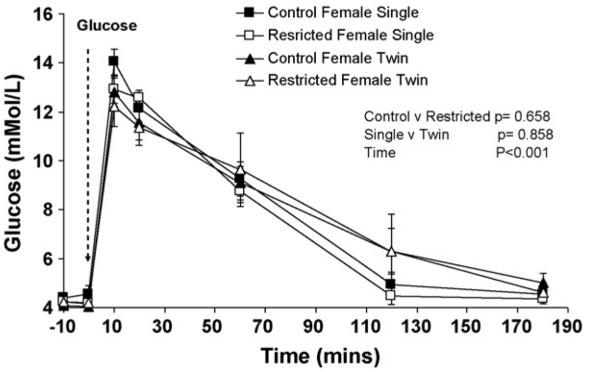

Fig. 2. Plasma glucose concentrations in female lambs at 10 weeks of age plotted relative to an intravenous infusion of glucose (0.5 g/kg body weight in saline; 50%, w/v). Single (square symbols) or twin (triangle symbols) female lambs were born to ewes fed a control diet (1.1× maintenance; closed symbols) or a restricted diet (0.7× maintenance; open symbols) from 28 days before to 7 days after conception.

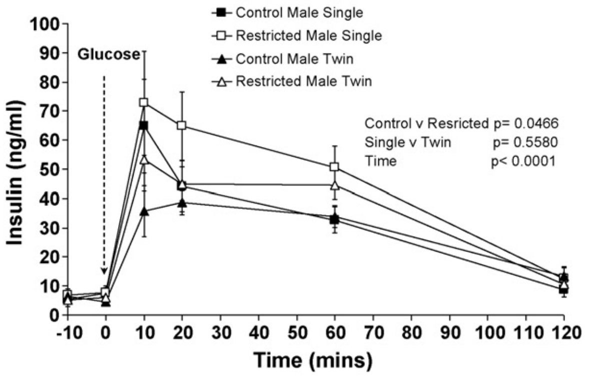

Fig. 3. Plasma insulin concentrations in male lambs at 10 weeks of age plotted relative to an intravenous infusion of glucose (0.5 g/kg body weight in saline; 50%, w/v). Single (square symbols) or twin (triangle symbols) male lambs were born to ewes fed a control diet (1.1× maintenance; closed symbols) or a restricted diet (0.7× maintenance; open symbols) from 28 days before to 7 days after conception.

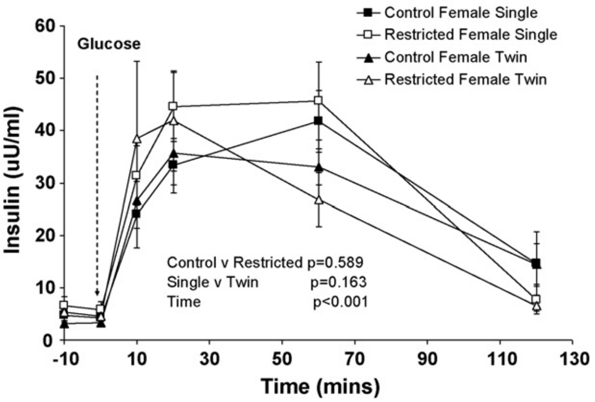

Fig. 4. Plasma insulin concentrations in female lambs at 10 weeks of age plotted relative to an intravenous infusion of glucose (0.5 g/kg body weight in saline; 50%, w/v). Single (square symbols) or twin (triangle symbols) female lambs were born to ewes fed a control diet (1.1× maintenance; closed symbols) or a restricted diet (0.7× maintenance; open symbols) from 28 days before to 7 days after conception.

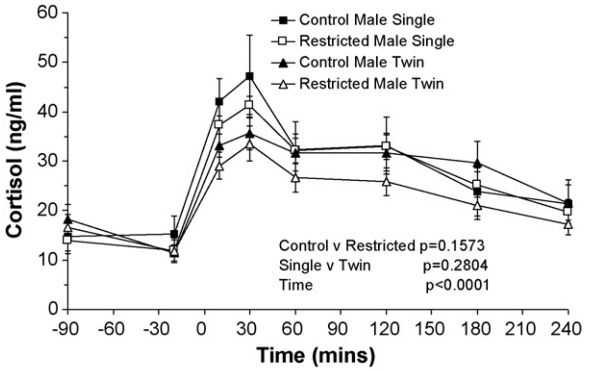

Fig. 5. Plasma cortisol concentrations in male lambs at 10 weeks of age plotted relative to an intravenous infusion of CRH (0.5 μg/kg body weight in a 5 μg/ml saline solution). Single (square symbols) or twin (triangle symbols) male lambs were born to ewes fed a control diet (1.1× maintenance; closed symbols) or a restricted diet (0.7× maintenance; open symbols) from 28 days before to 7 days after conception.

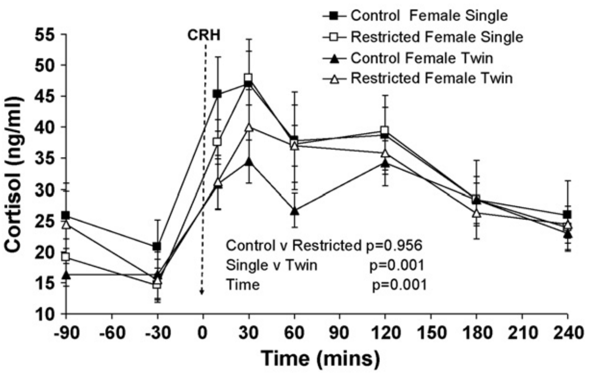

Fig. 6. Plasma cortisol concentrations in female lambs at 10 weeks of age plotted relative to an intravenous infusion of CRH (0.5 μg/kg body weight in a 5 μg/ml saline solution). Single (square symbols) or twin (triangle symbols) female lambs were born to ewes fed a control diet (1.1× maintenance; closed symbols) or a restricted diet (0.7× maintenance; open symbols) from 28 days before to 7 days after conception.

to a CRH challenge than the single group ($P = 0.001$; Fig. 6).

4. Discussion

Reduced growth in utero is associated subsequently with type II diabetes and impaired glucose tolerance in adult humans (recently reviewed by Morrison et al., 2010). Prenatal exposure to the Dutch famine, especially during late gestation, was linked to decreased glucose tolerance in adults aged about 50 years (Ravelli et al., 1998). In ewes, severe (40–50% restriction) and prolonged undernutrition early in gestation (to 30 days gestation or longer) has also been shown to alter the glucose–insulin metabolism in fetuses late in gestation (Oliver et al., 2001) and in offspring at 2 (Ford, 2007) and 12 (Gardner et al., 2005) months of age. We have now extended these findings to show that a mild (70% maintenance requirements) and short period of periconceptional undernutrition (ending on Day 7 of gestation) resulted in lower basal plasma glucose concentrations in single male offspring at birth compared to single control animals. This could indicate a lower glucagon reserve during parturition resulting in lower glucose levels at birth in this group. Also, at 10 weeks of age, male lambs born to nutritionally restricted mothers had a higher insulin response to a glucose challenge compared to the control group. It has been suggested that hyperinsulin response in infancy is caused by dysregulation of the apoptotic wave in β cells that matures the neonatal pancreas (Kassem et al., 2000). Thus, the hyperinsulinaemia observed in the male offspring of nutritionally restricted ewes at 10 weeks of age may also reflect dysregulation of pancreatic development caused by maternal periconceptional undernutrition. In contrast to the finding in males, the glucose–insulin system in female offspring was not affected by treatment. Hence, from studies in the literature it seems that maternal nutrition during the entire period encompassing preconception to the end of gestation has consequences for offspring glucose–insulin metabolism and implications for diabetes in later life but our study shows that short periods of maternal undernutrition and weight loss about the time of conception have lasting effects on the glucose–insulin system in males and not females.

The question still remains as to which mechanisms are responsible for differences seen in the glucose/insulin metabolism in offspring born to nutritionally restricted mothers. There is a growing body of evidence to show that early nutrition alters the epigenetic state of the embryo or fetal genome (Wu et al., 2006). Studies from in vitro embryo culture have shown that the methylation status of genomically imprinted genes, including IGF-II, H19 and IGF-2R can be altered with consequences for subsequent organ growth and function (Young et al., 2001; Waterland and Jirtle, 2004). In addition, there is now good evidence to show differences between male and female embryos in their growth rates, metabolism, gene expression levels and epigenetic status and a suggestion that under the same stress conditions male embryos are more vulnerable than female embryos (Gutierrez-Adan et al., 2006). The animal model described here could be exploited to further explore the molecular and cellular mechanisms which underpin sex dependent developmental and functional changes and the physiological consequences of poor maternal nutrition.

A number of studies have shown that maternal undernutrition during gestation negatively impacts the HPA axis in late gestation fetuses (Hawkins et al., 1999, 2001; Edwards et al., 2002) and young lambs (Hawkins et al., 2000; Chadio et al., 2007). In addition, maternal undernutrition around the time of conception results in a greater cortisol response to a CRH challenge in twin but not singleton fetuses in late gestation (Edwards et al., 2002). Periconceptional undernutrition from 14 days before conception until 70 days gestation resulted in reduced HPA response to administration of CRH and arginine vasopressin (AVP) in fetuses at 128 days of gestation. However, in contrast, restricted lambs had an elevated response compared to control lambs at 12 weeks of age (Hawkins et al., 2000). Our studies have extended these findings to show that the response of male lambs born to ewes that were nutritionally restricted at the time of conception, only tended to be lower than control lambs. It is possible that undernutrition about the time of conception is not sufficient to alter the HPA axis right through to postnatal life but it could be that the HPA axis is sensitive to a nutritional insult between Day 7 and Day 70 of gestation in order for negative outcomes to be manifest in adulthood. We conclude that maternal undernutrition around the time of conception has a minimal effect on the cortisol response to a CRH challenge (the HPA axis) in young lambs and this is in contrast to more profound effects on the functioning of the HPA axis in the offspring from ewes who were undernourished later in gestation (reviewed above).

In summary, maternal undernutrition around the time of conception (28 days before to 7 days after conception) resulted in single male lambs having lower glucose concentrations at birth and both single and twin male lambs having a greater insulin response to a glucose challenge at 10 weeks of age than control animals. The response of female lambs to a glucose challenge was not significantly different to control animals. In addition, maternal nutrition did not affect the response of either sex to CRH challenge. We conclude that a short period of maternal undernutrition about the time of conception did not affect the HAP axis of offspring but that there was a significant negative effect on the glucose–insulin system of male but not female offspring.

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Conflict of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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Social rank during pre-pubertal development and reproductive performance of adult rams

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ABSTRACT

Reproductive performance of rams when they were 1.5 and 2.5 years old during two breeding seasons was investigated. The objective was to compare the reproductive performance of the rams on the basis of their social ranks, which had been scored for each subject during the lamb development stage. The subjects were aged matched Corriedale × Milchschaf rams ($n = 12$: all singles born during the same week from multiparous ewes) were used. Data were collected from the five rams with greater (GR) and lesser (LR) social rank scores when lambs. These data included body weight, scrotal circumference, sexual behavior toward estrual ewes (e.g. incidence of ano-genital sniffing, lateral approaches, Flehmen, mount attempts, actual mounts, and mounts with ejaculations, and ejaculation/total mount ratio) during a 30-min period, and semen variables (semen volume, mass motility, individual sperm motility and total number of spermatozoa in the ejaculate). During the first breeding season, GR rams were heavier (62.9 ± 2.7 kg) than LR rams (69.4 ± 3.0 kg; $P < 0.0001$). There were no differences in scrotal circumference or testosterone concentration measures. Semen volume was greater in GR rams (0.5 ± 0.1 mL compared to 0.7 ± 0.1 mL for GR and LR rams; $P < 0.004$), but it tended to have a greater percentage of abnormal spermatozoa. Although some courtship behaviors were more frequent in LR rams (lateral approaches and Flehmen) and ejaculation/total mount ratio of LR rams also tended to be greater, mounting activity (mount attempts and mounts) was more frequent in GR rams. During the second year, GR rams still tended to be heavier (83.3 ± 2.0 kg compared to 85.9 ± 2.4 kg; $P = 0.09$) but had smaller scrotal circumference than LR rams (37.5 ± 0.2 cm compared to 36.5 ± 0.6 cm; $P < 0.045$). Semen characteristics were not different for the rams, but mass motility tended to be greater for LR rams. Generally, LR rams displayed more lateral approaches, showed Flehmen more often, mounted with ejaculation more often, and tended to have a greater mount with ejaculation/total mounts ratio. Conclusions were courtship of rams may differ in line with the social status they had as lambs although there are not clear effects on other sexual behaviors or semen characteristics.

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1. Introduction

The social environment in which male lambs are reared appears to influence some aspects of reproductive behavior. For example, continuous exposure to estrual ewes during the first 6 months of life induces an increase in testosterone concentrations and in testicular size (Illius et al., 1976a). Yearling rams exhibited enhanced sexual performance and greater sexual interest in females where they had previous direct contact with females at 7–9 months of age (Price et al., 1991, 1994).

Rams that were reared in male groups, without contact with females, have less interest in females (Zenchak and Anderson, 1980). According to Welch (1972) (cited by

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Zenchak and Anderson, 1980), only 30% of the rams that were reared in isolation from females subsequently mated, compared to 82% of the rams reared in contact with males and females. Similarly, De Montigny and Lequenne (1975) (cited by Orgeur et al., 1990) reported difficulty associated with collecting semen from rams that had not come in contact with females prior to 5 months of age. Stellflug and Lewis (2007) demonstrated early exposure of ram lambs to estrual ewes improves sexual performance in serving capacity tests of adult rams.

However, there is no information in relation to the influences of other social stimuli during rearing on adult life. In adult rams, lesser-ranked males have limited access to receptive females (Preston et al., 2003), as sexual activity is frequently disrupted by other rams (Hogg, 1984). In pen tests, presence of dominant rams affects the sexual behavior of subordinate rams (Lindsay et al., 1976; Synnott and Fulkerson, 1984; Tilbrook et al., 1987; Ungerfeld and González-Pensado, 2008a). Similarly, social hierarchies affect reproductive development of male lambs (Ungerfeld and González-Pensado, 2008b). Lambs with greater social ranking had greater body weight and scrotal circumference at earlier ages than lesser-ranked lambs, and semen production and sexual behavior also occurred earlier in lambs with greater social ranking than in lesser-ranked lambs (Ungerfeld and González-Pensado, 2008b). However, it remains to be determined if social rank during rearing affects not only development rate, but also, as happens with female presence, affects the adult male reproductive performance as well. If with a lesser social rank of lambs affects negatively the adult rams' reproductive activity, practical management to avoid these effects should be developed. For example, if rams with a greater social rank have priority access to food, it may be possible to feed them separately from lambs with a greater social rank.

Therefore, objectives of the present study were to compare the reproductive performance (semen production and sexual behavior) of rams during the breeding season in relation to the social rank they had during development as lambs. Therefore, the experiment was performed with the same rams used during their first 9 months in Ungerfeld and González-Pensado (2008b). Tests were performed when rams were 1.5 and 2.5 years old.

2. Materials and methods

2.1. Animals and management

For the experiment, the same rams previously used to determine the influence of social rank on male lamb development (Ungerfeld and González-Pensado, 2008b) were used. However, two rams were eliminated due to health problems, one medium-ranked (not considered for data analysis in the previous work) the first year, and one originally lesser-ranked during the second year. The 12 Corriedale × Milchschaf rams, which were all singles born the same week from multiparous ewes, were moved to the facilities of the Departamento de Fisiología, Facultad de Veterinaria, Montevideo, Uruguay (35°S) when they were 9 months old. Rams were maintained in a 10 m × 10 m pen during all the experiment, and received alfalfa hay, 500 g of concentrate per day and water was available *ad libitum*. The study was performed during the two following breeding seasons, when rams were 1.5 and 2.5 years old. Data were collected from the five rams that had greater (GR) and lesser (LR) social rank during the pre-pubertal stage.

2.2. First year trial (yearling rams)

The data were collected during the February–June period (mid-summer to the end of autumn). Social rank success index (Section 2.4) was determined in March. Rams were weighed and scrotal circumference was recorded every week by the same observer. Serum samples were obtained weekly by jugular venipuncture. Samples were allowed to clot for 1 h at room temperature before being centrifuged for 10–20 min, and stored at −20° C until assayed for testosterone. Testosterone was measured with a Count-A-Count (TKPG, Diagnostic Products Corporation) solid-phase kit. The detection limit of the assay was 0.5 nmol/L. The intra-assay coefficients of variation were all below 10%. Semen was collected every 2 weeks, and sexual behavior was also recorded every 2 weeks during the same period.

2.3. Second year trial (adult rams)

The data were collected from February 18 to April 30 (mid-summer to mid-autumn). Success index was determined during February. Rams were weighed and scrotal circumference was recorded weekly in the first 2 weeks of each month. Semen was collected, and sexual behavior toward estrual ewes, e.g. incidence of ano-genital sniffing, lateral approaches, Flehmen, mount attempts, actual mounts, and mounts with ejaculations, and ejaculation/total mount ratio during for a 30-min period was also recorded.

2.4. Social rank

As in Ungerfeld and González-Pensado (2008b), hierarchy was determined using the food competition test (Synnott and Fulkerson, 1984). Rams were used to this test, as it has been applied every 3 months during all their life as part of other experiments. After fasting the rams for 12 h, each dyad of rams was allowed to compete for feed in a bucket only large enough to accommodate one ram eating from the vessel at any one point in time. Paired encounters were performed each day with each ram tested once. Only one test was performed with each ram each day. When a ram was able to eat from the bucket for more than 1 min, it was considered to be the dominant individual of the pair (Lindsay et al., 1976). After testing every possible pair of rams, a success index based on the number of tests in which each ram was considered as dominant was calculated.

2.5. Semen collection and evaluation

To avoid differences provoked by rams' libido, semen was collected using an electroejaculator. The penis of the rams were grasped and held at the end of a calibrated centrifuge tube. Electrical stimulation was applied for intervals

Table 1
Body weight and reproductive performance of 1.5 years old rams according to the hierarchical position they had as lambs: LR (*n* = 5), were lesser-ranked when lambs; GR (*n* = 5), were greater-ranked when lambs.

	LR	GR	*P*
Body weight (kg)	62.9 ± 2.7	69.4 ± 3.0	<0.0001
Scrotal circumference (cm)	31.4 ± 0.9	31.8 ± 1.0	ns
Testosterone concentrations	17.8 ± 7.9	19.4 ± 6.0	ns
Semen characteristics			
Volume (mL)	0.5 ± 0.1	0.7 ± 0.1	0.004
Mass motility	2.9 ± 0.2	3.1 ± 0.2	ns
Individual motility (%)	71.9 ± 11.3	79.9 ± 3.9	ns
Total spz ($\times 10^6$)	2482 ± 503	2968 ± 331	ns
Abnormal spz (%)	9.6 ± 2.3	14.0 ± 1.8	0.07
Sexual behavior			
Courtship onset (s)	0.0 ± 0.0	0.0 ± 0.0	ns
Ano-genital sniffing	23.1 ± 1.3	21.9 ± 1.5	ns
Lateral approaches	101.4 ± 7.3	75.9 ± 5.8	0.001
Flehmen	3.8 ± 0.4	2.6 ± 0.4	0.01
Mount attempts	4.6 ± 0.8	6.8 ± 0.9	0.04
Mounts	6.2 ± 0.9	10.1 ± 1.2	0.005
Mounts with ejaculation	2.6 ± 0.3	2.6 ± 0.3	ns
Mounts with ejaculation/total mounts	0.30 ± 0.03	0.23 ± 0.03	0.08
Time to first mount with ejaculation (s)	166.8 ± 45.6	172.2 ± 58.6	ns

of 3–5 s and alternated with rest periods of similar duration until semen was obtained. Semen volume, mass motility (scales 0–5) and individual sperm motility were recorded according to Evans and Maxwell (1987). Sperm concentration was recorded and total number of spermatozoa in the ejaculate was calculated.

2.6. *Sexual behavior*

The sexual behavior of the rams was tested in a 5 m × 5 m pen with an estrual ewe. One ovariectomized ewe was induced to estrus with an hormonal treatment (5–6 days of intravaginal sponges impregnated with medroxi-progesterone acetate, and 12 h injections of estradiol benzoate) for each weekly test. The incidence of ano-genital sniffing, lateral approach, Flehmen response, mount attempts, mounts, and mounts with ejaculations were recorded for a 30-min period. The total mount number (mounts + mounts with ejaculations) and the ejaculation/total mount ratio was calculated.

2.7. *Statistical analysis*

Success indexes from both LR and GR rams were compared with an ANOVA. Data were compared with a hierarchical ANOVA, considering each time point as the subgroups that generated the data for each animal.

3. Results

3.1. *First year trial (yearling rams)*

Success index did not differ between LR (0.45 ± 0.16) and GR (0.55 ± 0.10). GR rams were heavier than LR rams. There were no differences in scrotal circumference or testosterone concentrations. Semen volume was greater in GR rams, but this semen tended to have a greater percentage of abnormal spermatozoa (Table 1).

Although some courtship behaviors were more frequent in LR than GR (lateral approaches and Flehmen), mounting activity (mount attempts and mounts) was more frequent in GR ($P = 0.04$ and 0.005 respectively). The ejaculation/total mount ratio of LR tended to be greater (Table 1).

3.2. *Second year trial (adult rams)*

Success index did not differ between LR (0.42 ± 0.07) and GR (0.55 ± 0.14) rams. GR rams tended to be heavier ($P = 0.09$), and had smaller scrotal circumference ($P = 0.045$) than LR rams. Semen characteristics were not different for GR and LR rams, but mass motility tended ($P = 0.08$) to be greater in semen from LR than GR rams. There were differences in sexual behavior: LR rams displayed more lateral approaches ($P < 0.001$), showed Flehmen more times ($P = 0.03$), mounted with ejaculation more times ($P = 0.007$), and tended to have a greater mount to ejaculation/total mounts ratio ($P = 0.08$). Number of mount attempts was, however, greater in LR ($P = 0.02$) (Table 2).

4. Discussion

Overall, the evidence suggests that the ram reproductive performance may be related to the social rank of the individual ram during the pre-pubertal stage. This expands previous concepts relating social environment during male lamb rearing and ram sexual behavior. Contact with females during rearing positively influences rams serving capacity as adults (Price et al., 1991, 1994; Stellflug and Lewis, 2007; Zenchak and Anderson, 1980). Presence of dominant individuals, however, delays reproductive development (Ungerfeld and González-Pensado, 2008b), and also affects sexual behavior as adults. Thus, the rams with a lesser social rank as lambs courted more but mated less than those that were greater social rank. Although 1 year later, rams that had been lesser-ranked had increased serving capacity, they still had a greater number of courtship events.

Table 2
Body weight and reproductive performance of 2.5 years old rams according to the hierarchical position they had as lambs: LR ($n = 4$), were lesser-ranked when lambs; GR ($n = 5$), were ranked as having greater social status as lambs.

	LR	GR	*P*
Body weight (kg)	83.3 ± 2.0	85.9 ± 2.4	0.09
Scrotal circumference (cm)	37.5 ± 0.2	36.5 ± 0.6	0.045
Semen characteristics			
Volume (mL)	1.0 ± 0.1	1.2 ± 0.1	ns
Mass motility	3.3 ± 0.3	2.8 ± 0.2	0.08
Individual motility (%)	68.6 ± 4.7	64.8 ± 3.6	ns
Total spz ($\times 10^6$)	1700 ± 246	2371 ± 441	ns
Abnormal spz (%)	21.1 ± 3.5	18.9 ± 2.0	ns
Sexual behavior			
Courtship onset (s)	0.0 ± 0.0	0.0 ± 0.0	ns
Ano-genital sniffing	10.8 ± 1.4	9.0 ± 1.4	ns
Lateral approaches	112.5 ± 8.7	74.0 ± 6.5	<0.001
Flehmen	3.2 ± 0.7	1.7 ± 0.4	0.03
Mount attempts	6.9 ± 1.7	13.0 ± 2.2	0.02
Mounts	4.8 ± 1.1	4.3 ± 0.8	ns
Mounts with ejaculation	2.8 ± 0.3	1.7 ± 0.3	0.007
Mounts with ejaculation/total mounts	0.5 ± 0.1	0.3 ± 0.1	0.08
Time to first mount with ejaculation (s)	205 ± 75	328 ± 98	ns

The social rank of male lambs during pre-pubertal development affected reproductive performance as adult rams. However, although reproductive performance was clearly greater in lambs with a greater social ranking than in lesser-ranked lambs (Ungerfeld and González-Pensado, 2008b), results as adults differ. Frequency of courtship behavior events was greater in LR rams during both breeding seasons. This is similar to what we observed in adult rams: when tested alone with estrual ewes, rams with a greater social ranking displayed less courtship events (Ungerfeld and González-Pensado, 2008a). Present results indicate reproductive strategies that determine differential access to females is affected by social rank in earlier life stages. Differences in courtship variables were maintained as adults although social rank did not differ between GR and LR rams as adults. Mount activity and mount effectiveness, however, changed over time, suggesting it may be a more flexible behavior, and rams may adapt with changes in age.

There were no differences in testosterone serum concentrations with social status. This agrees with observations of Illius et al. (1976b), who reported that testosterone pattern in male rams until 21 months of age was not modified by the social environment (isolated, in male groups, or in sex-mixed groups) and Kridli and Al-Yacoub (2006) where sexual environment in which male lambs were reared (with or without ewes) did not influence testosterone concentrations. Perkins and Fitzgerald (1994) in adult rams, and Ungerfeld and González-Pensado (2008b) in lambs, reported that testosterone concentrations were similar between males that displayed greater or lesser sexual behavior events. Pelletier et al. (2003) reported a relationship between social rank and testosterone production, but this difference was not detectable when age was taken into account. As in the present experiment, all rams were born the same week, possible effects of age on social rank, and therefore in testosterone secretion, could not be detected. Therefore, the differences in sexual behavior observed are not related to a greater production of testosterone, but may be related to differences in sensitivity to testosterone determining that with similar concentrations some rams display greater sexual behavior and have greater serving capacities.

There were no important differences in semen production as related to social rank. Aguirre et al. (2007) reported greater semen volume and sperm concentration in rams with greater social ranking than in lesser-ranked rams, but rams were evaluated in small groups, in which hierarchical effects may be greater. In the present experiment, only long-term effects of hierarchy in a lamb's life were detected. In wild rams it has been proposed that although copulation rate is not affected by the length of the rut period, there is a decline in the proportion of lambs sired by larger males as the rut progresses (Preston et al., 2001). Moreover, ejaculates from males with a greater copulation rate had fewer spermatozoa per ejaculate, with a greater proportion of abnormal spermatozoa (Preston et al., 2001). This lesser amount of sperm may be caused by greater ejaculation rates, as shown in domestic rams (Thwaites, 1994). However, in the present experiment rams only mated ewes during the sexual tests, so sexual activity did not have a direct influence in semen characteristics. Although hierarchical positions (Ungerfeld and González-Pensado, 2008b) or the presence of ewes (Casteilla et al., 1987) advances semen production in pubertal lambs, lamb social rank does not have consequences on semen quality of adult rams.

Although lambs with a greater social rank had greater body weights than lesser-ranked lambs (Ungerfeld and González-Pensado, 2008b), differences were maintained the first year, but only tended to be so during the second year. This agrees with pervious findings, as body weight differed in lambs (Ungerfeld and González-Pensado, 2008b), but not in adult rams (Ungerfeld and González-Pensado, 2008a). One possible explanation for the lack of difference is that although body mass is closely related to social rank in adult wild rams (Pelletier and Festa-Bianchet, 2006) domestication implies lesser pressures for animals, where animals require less time and energy to access food and appropriate diet, are provided with protection from predators and appropriate health and welfare management (Price, 2002). It should be considered that yearling rams not fully matured, as body weights were 75% to 80% of mature body weight. Although growth rate may have been less in LR rams, probably growth time was longer in LR rams allowing them to compensate in growth later in life. Similarly, Festa-Bianchet et al. (2000) observed that male lamb body mass was not correlated with adult body mass in bighorn rams. Therefore, the findings from the current study suggest that social rank and body weight in sheep are possibly more closely associated during the pre-pubertal development stage than during the later stages of adulthood.

Overall, courtship patterns of mature rams may differ with social status as lambs, there are not clear effects on other sexual behaviors or semen characteristics as mature rams.

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Alterations in mitochondrial function and spermatozoal motility in goat spermatozoa following incubation with a human lysozyme plasmid

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ABSTRACT

Spermatozoa mediated gene transfer has become a promising technology to generate transgenic animals with disease resistance. However, exogenous DNA invasion may cause changes in spermatozoon natural defense system which result in spermatozoon dysfunction. The objective of this study was to investigate the changes of mitochondrial function and motility in goat spermatozoa after pre-incubation and incubation with and without the human lysozyme plasmid pFLAG-hLY. The results demonstrated that human lysozme plasmid pFLAG-hLY could bind to the surface of the spermatozoon membrane at 186,000 copies/spermatozoa and incorporate to spermatozoon nucleus at 78 copies/spermatozoa after incubation. However, the treated spermatozoon samples showed a significant lower motility (29.7 ± 2.2% vs. pre-incubation control 48.0 ± 1.4% and incubation control 54.5 ± 1.5%, $P < 0.05$), and percentage of rapid progressive motile spermatozoa (16.4 ± 2.4% vs. pre-incubation control 31.4 ± 0.6% and incubation control 37.0 ± 0.5%, $P < 0.01$). Meanwhile, the incubation with plasmids caused significant reduction of mitochondrial membrane potential (31.44 ± 2.17% vs. pre-incubation control 51.79 ± 2.08% and incubation control 58.81 ± 1.76%, $P < 0.05$). In addition, dichlorofluorescin relative fluorescence intensity (32.81 ± 2.41%) and malondialdehyde levels (2.18 ± 0.21 nM/10^8 spermatozoa), which represents mitochondrial function, showed a significant increase after incubation ($P < 0.05$). The cytochrome *c* release from the mitochondrial inner membrane space, and enzymatic activities of caspase-3 (0.086 ± 0.024) and caspase-9 (0.083 ± 0.019) ($P < 0.05$) also increased, in which resulted in spermatozoon dysfunction. In conclusion, this study confirmed that goat spermatozoa could capture human lysozyme plasmid pFLAG-hLY, but the incubation with the plasmids resulted in a decrease of spermatozoa motility and partial rupture of mitochondrial membrane, and further prompted the expression of cytochrome *c*, and generation of oxidative stress in vitro and finally led to spermatozoon dysfunction.

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1. Introduction

Transgenic animals have a great potential to increase animal disease resistance and improve livestock breeds by introducing specific new genetic material into animal cells or organism. Goat is a potentially appropriate species for the production of large amounts of recom-

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binant proteins from transgenic organisms (Houdebine, 2000, 2002). For example a recent study (Li et al., 2009) has indicated that human lysozyme is an important recombinant protein for innate immunity and its antibacterial activity is many times greater than that of other animal species milk lysozyme. Thus, we consider that, by means of transgenic technology, the human lysozyme gene may be used to reduce the high incidence of mastitis infection.

Currently, transgenic animals may be conventionally produced through various developed transgenic technologies, such as pronuclear microinjection (Jon and Frank, 1983), nucleus transfer (Karatzas et al., 1999), sperm-mediated (Lavitrano et al., 1992) and embryonic stem cells (Richard et al., 1984). One alternative method is sperm-mediated gene transfer (SMGT), which uses spermatozoon as a natural vector to introduce new genes into animals. In contrast with other established methods, this method has the advantages of simplicity and cost-effectiveness (Smith, 2002; Garcia-Vazquez et al., 2009). Currently, this gene transfer technique can be widely applied in many species (Garcia-Vazquez et al., 2009) and has been efficient in successfully producing large transgenic offspring including pigs (Aitken et al., 1998) and cattle (Shemesh et al., 2000; Donovan et al., 2005; Wall et al., 2005). So far, it has been found that spermatozoa of many animal species are able to uptake spontaneously exogenous DNA of mouse (Lavitrano et al., 1992), rabbit (Fan and Watanabe, 2003), ram and buck (Castro et al., 1992) and bull (Schellander et al., 1995). However, it has been reported that spermatozoon interactions with exogenous DNA can trigger endogenous nuclease(s) which cleave exogenous and genomic DNA (Spadafora, 1998), and induced a significant decrease in spermatozoa motility (Schellander et al., 1995). Also, nuclease activation may indicate the presence of a significant increase in spermatozoon genomic DNA damage after the binding of exogenous DNA (Kang et al., 2008). Alternatively, endonuclease activation may result in the disruption of spermatozoon DNA repair mechanisms (Anzar and Buhr, 2006) and increased susceptibility to oxidative stress and the presence of apoptotic-like process.

In last decade, many experiments in SMGT have attempted to identify the effect of different treatments including incubation time, temperature, spermatozoon selection and optimization of DNA uptake on spermatozoa motility (Lavitrano et al., 2003; Wu et al., 2009). However, there has been no examination of mitochondrial function alterations of spermatozoa after spermatozoa with exogenous DNA. The objective of the present study was to investigate the effect of spermatozoon incubation with plasma pFLAG-hLY (human lysozyme plasmid expressing the FLAG-tagged) on spermatozoa motility and mitochondrial functions.

2. Materials and methods

2.1. Reagents and media

Except for otherwise stated, all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) with the highest available purity. The basic dilutions and culture media were cell culture grade routinely used in our laboratory.

2.2. Preparation and measurement of goat spermatozoa

2.2.1. Semen collection and processing

Five clinically healthy and fertile Saanen bucks at 2–3 years old were selected from China's first animal cloning base in Yangling, Shaanxi Province. A total of 160 ejaculates (32 from each animal) were obtained, frozen and thawed (Hidalgo et al., 2007). Aliquots of each spermatozoon sample were assessed subjectively for the following variables: volume, concentration, morphology and motility.

The samples of frozen–thawed semen in this study were selected at 300 × *g* with 40:80% PureSperm density gradient (40/80, Nidacon International, Goteborg, Sweden) for 20 min at room temperature (Ricci et al., 2009). After the swim-up technique, motile spermatozoa were suspended in Tyrode's bicarbonate buffer-HEPES (TALP-HEPES, 114 mM NaCl, 25 mM $NaHCO_3$, 2.10 mM $CaCl_2.2H_2O$, 3.20 mM KCl, 0.34 mM $NaH_2PO_4{\cdot}H_2O$, 1.50 mM $MgSO_4{\cdot}7H_2O$, 10.07 mM HEPES and 50 μg/ml gentamycine) described in detail (Nedambale et al., 2006) with 5 mg/ml BSA fraction V. The viable spermatozoa were suspended for further experiments.

2.2.2. Detection of spermatozoa motility

Upon incubation, spermatozoa motility (percentage) was assessed immediately under a light Olympus BX 51 microscope (Tokyo, Japan) at a 37 °C warming plate. The percentage of rapid progressive motile spermatozoa (RPMS) was estimated under a phase-contrast microscope at 200× magnification by two independent observers.

2.3. The pFLAG-hLY plasmid and incubation with spermatozoa

The pFLAG-hLY plasmid was constructed in our Laboratory and was linearized by digesting with HindIII and purified from solution using a plasmid purification kit from Promega Corporation (Madison, WI, USA). The plasmid DNA was stored at 0.5 μg/ml in Tris–EDTA solution.

Following the preliminary protocol, the selected samples (1×10^6/ml) were incubated with or without linearized pFLAG-hLY (0.05 μg/ml) in 1 ml of modified TALP-Fert medium (114 mM NaCl, 25 mM $NaHCO_3$, 2.10 mM $CaCl_2.2H_2O$, 3.20 mM KCl, 0.34 mM $NaH_2PO_4{\cdot}H2O$, 1.50 mM $MgSO_4{\cdot}7H_2O$, 0.222 mg/ml sodium pyruvate, 20 μM penicillinamine, 10 μM hypotaurin, 2 μM epinephrine, 6 mg/ml fatty acid free BSA, 50 μg/ml gentamycine and 1 μg/ml heparin) for 30 min at 37 °C and 5% CO_2 using 8 μl of FuGene 6 transfection Reagent (Roche, Germany). In the pre-incubation control group, the selected samples were suspended only with TALP-Fert medium.

2.4. Real-time PCR quantification in spermatozoa incubated with pFLAG-hLY

2.4.1. DNA isolation from whole spermatozoa

Spermatozoon genomic DNA extraction was performed using a QIAamp DNA mini kit (QIAGEN, Germany) according to the manufacturer's instruction (Hoelker et al., 2007). Briefly, after the spermatozoon sample incubated with pFLAG-hLY was centrifugated and washed in TALP-HEPES buffer, 200 μl sample was added with 20 μl of QIAGEN protease (stock solution) and 4 μl of RNase A (stock solution), and lysed in buffer AL (stock solution) for 30 min at 56 °C. Each sample was pulse-vortexed for 15 sec and centrifuged at 12,000 × *g* for 5 min. The DNA was further purified with DNA fragment purification kit from TaKaRa (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). DNA quality was examined by a 1.0% (w/v) agarose gel electrophoresis and DNA amount was quantified by measuring absorbance at 260 nm using a computerized Microplate Reader 550 from the Bio-Rad Company (Hercules, CA, USA). The recovered DNA was stored in Tris-EDTA buffer (pH 8.0) at −20 °C and used as template for quantitative real-time PCR.

2.4.2. DNA isolation from spermatozoa nuclei

For spermatozoa nuclei isolation, the sample was incubated with pFLAG-hLY and centrifuged and washed twice with 150 mM NaCl at 600 × *g* for 3 min, and finally was resuspended at 1×10^8/ml in the nuclei isolation buffer (pH 9.0, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF and 1 μg/ml pepstatin A). The spermatozoon suspension was collected by centrifugating at 600 × *g* for 3 min and washed three times with the isolation buffer (fresh DTT 25.4 mM, final concentration) and incubated for 30 min on ice. Afterwards, the sample was added with CTAB buffer (10% CTAB, 10 mM DTT) and was incubated again for 60 min on ice. The obtained spermatozoon nuclei were immediately centrifugated at 10,000 × *g* for 5 min and washed twice with the nuclei isolation buffer as template for quantitative real-time PCR.

2.4.3. Real-time PCR quantification

Quantitative analyses of the human lysozyme (hLY) gene was carried out by comparing the copy number of plasmid with the copy number of samples as previously described (Hoelker et al., 2007). The concentration of purified DNA samples was quantified fluorometrically using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, USA) and a VersaFluor fluorometer (Bio-Rad). The same amount of DNA was used in each Real-Time PCR. SYBR Green as a specific dye was used for quantitative Real-Time PCR reaction, in triplicate, in an ABI Prism 7500 Sequence Detection System. In the quantification of bind and internalization of plasmid pFLAG-hLY by goat spermatozoa, primer sets were designed using Primer 5.0 and criteria used during primer design were that primers have predicted Tm = 60.0 °C and that they generate to amplify a 130-bp partial gene fragment and primers bind to the conserved regions of human lysozyme gene (accession no.: NM000239). The primers containing the FLAG epitope were designed as follows: forward primer 5′-CAAGGATGACGACGATAAGATGAAG-3′ and reverse primer 5′-GCCATCCATTCCCAATCTTTTC-3′. The PCR amplifications were carried out in a 20 μl reaction volume containing 10.0 μl of SYBR® Green Master Mix (PE Biosystems), 6.8 μl of double-distilled water, 0.6 μl of 10 μM forward, 0.6 μl of 10 μM reverse primers and 2.0 μl of the purified DNA solution (10–20 ng). The thermal profile for SYBR RT-PCR was 50 °C 2 min, 95 °C 10 min followed by 50 cycles of 95 °C 10 s and 60 °C 1 min. The CT value was used to determine the copy numbers of human lysozyme gene in the unknown spermatozoon samples incubated with pFLAG-hLY based on the standard curve. The real-time standard curves were derived from a serial of linearized plasmid dilutions containing between 10^9 and 10^1 target gene copies calculated directly from the concentration of extracted standard plasmids. The copy numbers of standard plasmids and human lysozyme genes of the unknown sample from the pFLAG-hLY groups were determined using a standard curve. All reactions were repeated at least 3 times independently to ensure the reproducibility of the results

2.5. Mitochondrial membrane potentials ($\Delta\Psi m$)

The $\Delta\Psi$m changes of treated spermatozoa were evaluated using a FACSCalibur flow cytometer (BDBioscience, San Jose, CA) and a JC-1 (lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, the spermatozoa sample (2×10^6/ml) was stained with 28 μl of JC-1 (stock solution) and 5 μl of PI (stock solution) in 100 μl (final volume) of TALP solution. After incubation at 37 °C in the dark for 30 min, the samples were collected and placed on ice using the method previously described (Pant et al., 2008). High membrane potential was associated with emission at 590 nm (red), and low membrane potential at 530 nm (green) when spermatozoa were excited at 488 nm. $\Delta\Psi$m was determined by a ratio of fluorescence intensity at 590 nm to that at 530 nm. Data were processed by using the CellQuest program (BDBioscience, San Jose, CA). Spermatozoa were treated with 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which is a protonophore which can cause dissipation of $\Delta\Psi$m, were used as a negative control.

2.6. Assay of reactive oxygen species (ROS) generation

The levels of intracellular reactive oxygen species were estimated using the membrane-permeable fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA) and a FACSCalibur flow cytometer. The treated spermatozoa (1×10^6/ml) before the end of each experiment and, in three groups, were incubated in 1 ml of mPBS containing DCFH-DA dye (10 μM) at 37 °C for 15 min. Propidium iodide (PI) was then added to DCFH-DA-loaded cell suspensions at a final concentration of 10 μg/ml for approximately 1 min before measurement. The DCHF-DA probe passively diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped inside cells. The ROS produced by spermatozoa oxidizes DCHF to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Then, spermatozoa fluorescence was measured and analyzed by flow cytometry. When DCF was oxidized by ROS, they emit green fluorescence at 510–540 nm after excitation at 488 nm with an argon ion laser. The intracellular ROS levels were expressed as DCF relative fluorescence intensity per 10^6 cells from independent experiments.

2.7. Determination of lipid peroxidation

The level of malondialdehyde (MDA) production, a measure of lipid peroxidation, was measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the spermatozoon suspensions (approximately 5×10^8/ml) and the reaction mixture containing 0.5 ml thiobarbituric acid reagent (1:1, v:v, mixture of 0.67% thiobarbituric acid and acetic acid) were mixed, shaken, and heated at 95 °C for 40 min. After cooling, the tubes were centrifuged at $4000 \times g$ for 10 min at 4 °C and the supernatants were removed for the detection of lipid peroxidation. The absorbance of the clear supernatant was determined at 532 nm using a computerized microplate reader 550. The MDA content was expressed as nM/10^8 spermatozoa.

2.8. Cytochrome *c* release

Cytochrome *c* levels in both mitochondrial and cytosolic fractions were analyzed by western blotting (Hanzel and Verstraeten, 2009). The spermatozoa (5×10^7/ml) were harvested before the end of each experiment by centrifugation at $600 \times g$ for 10 min at 4 °C,then washed twice with TALP buffer. The spermatozoon pellets were then lysed by incubation at 56 °C overnight with gentle agitation in Tris buffer (pH 8.3) containing 0.45% NP-40, 0.45% Tween-20, 400 μg/ml proteinase K, 1 mM PMSF, 40 mM DTT, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin and 40 μg/ml bestatine. After centrifugation twice at $600 \times g$ for 15 min at 4 °C, the supernatants were again centrifuged twice at $7000 \times g$ for 20 min at 4 °C to obtain sediments considered as the mitochondria fraction. The mitochondrialis fractions were suspended in 10 mM Tris–HCl (pH 8.0) buffer containing 0.3 mM sucrose, 0.05% (v/v) Igepal, 5 mM $CaCl_2$, 10 μM Aprotinin, 10 μM Pepstatin, 10 μM Leupeptin and 1 mM PMSF. Cytosolic fractions were obtained by centrifugation at $100{,}000 \times g$ for 1 h at 4 °C. After centrifugation, the protein content of both fractions was measured using a Bradford protein assay kit (KeyGEN Biotech. Co. Ltd., Inc., Nanjing, China). Briefly, equal amounts of protein were subjected to SDS–PAGE, followed by transfer to PVDF membranes (Millipore, USA). Membranes were then blocked in 5% powdered non-fat milk in a Tris/Tween solution (20 mM Tris–HCl, pH 7.6, 0.1% Tween 20 in saline) for 2 h. The location and change in mitochondrial cytochrome *c* level probed with specific anti-cytochrome *c* antibodies (diluted at 1:500) from Cell Signaling (Abcam, USA). Following incubation with appropriate secondary anti-rabbit antibodies at a 1:5,000 dilution, immunoreactive protein bands were visualized by an enhanced chemiluminescence detection kit (Invitrogen, USA) and were recorded on X-ray film (Kodak, China). As control, the blots were reprobed with anti-β-actin antibody (KeyGEN Biotech. Co. Ltd., Nanjing, China) at 1:1500 dilutions. Finally, the membranes were exposed for varying periods ranging from 10 s to 5 min. The band signal intensities were quantified with a Bio-Rad GS-800 Calibrated Densitometer and Bio-Rad personal computer analysis software (Bio-Rad, USA).

2.9. Caspase-3 and -9 activities

Activities of caspase-3 and -9 of spermatozoa samples were determined at an absorbance (OD, optical density) at F405 nm using a caspase-3 and -9 Colorimetric Protease Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). 5×10^6/ml spermatozoon samples were washed with cold PBS by centrifugation at $600 \times g$ at 4 °C for 5 min, and then resuspended in lysis buffer in a HEPES buffer (pH 7.4) of 50 mM containing 0.1% Igepal, 1 mM DTT, 0.1 mM EDTA, and 150 mM NaCl, and were incubated on ice for 15 min. The lysate was centrifuged at $16{,}000 \times g$ at 4 °C for 15 min. Proteins content was measured in the supernatant using the Bradford method (Zor and Seliger, 1996) with bovine serum albumin as a standard, and the protein was adjusted to 2 μg/μl. Subsequently 80 μl reaction buffer, 10 μl of protein solution and 10 μl 2 mM caspase-3 substrate (acetyl-Asp-Glu-Val-Asp p-nitroanilide, Ac-DEVD-pNA) or caspase-9 substrate (Ac-Leu-Glu-His-Asp-paranitroaniline, Ac-LEHD-pNA) were mixed sequentiallyand the samples were incubated at 37 °C for 4 h. The cleavage and release of paranitroanilide (pNA) was qualified by determining the absorbance with a computerized microplate reader 550 (Bio-Rad, USA) at 405 nm using spectrophotometer, expressed as OD_{405} (test). A total of 90 μl of reaction buffer and 10 μl 2 mM of the corresponding substrate buffer were used for pre-incubationsand defined as OD_{405} (pre-incubation). The activities of caspase 3 and -9 were expressed as OD_{405} (test)/OD_{405} (pre-incubation). Assays were performed in duplicate, and at least three independent tests were performed.

2.10. Data analysis and statistics

Analysis of variance was applied to determine possible effects of incubation with pFLAG-hLY on mitochondrial function, oxidative damage and sperm characteristics. Data were analysed for main effects of the pre-incubation control, the incubation control and pFLAG-hLY groups using one-way analysis of variance (ANOVA) of SPSS for Windows 17.0 Software (SPSS, Inc. Chicago, IL, USA). The independent tests were repeated three times in each group. All the values are presented as mean ± standard error of the mean (SEM). Error bars represent the 95% confidence interval of the mean percentage. A probability (*p*) value of <0.05 or <0.01 was considered statistically significant (two-tailed).

3. Results

3.1. The ability of goat spermatozoa binding human lysozyme gene

To quantify the copy number of plasmid pFLAG-hLY binding to and internalizating into spermatozoon, a serials of dilutions of extracted genomic DNA or nucleus were assayed by primers (forward primer sequence, 5′-CAAGGATGACGACGATAAGATGAAG-3′; Reverse primer sequence, 5′-GCCATCCATTCCCAATCTTTTC-3′), the resultant CT value against the amount of plasmid DNA was

Table 1

Change of goat sperm motility and RPMS after incubation with pFLAG-hLY for 30 min.

Parameters	Pre-incubation control	Incubation control	pFLAG-hLY
Sperm motility (%)	48.0 ± 1.4^a	54.5 ± 1.5^a	29.7 ± 2.2^b
RPMS (%)	31.4 ± 0.6^a	37.0 ± 0.5^a	16.4 ± 2.4^c

a, b and a, c represent significant differences among groups (a, b: $P < 0.05$, a, c: $P < 0.01$). Results come from triplicate experiment average and standard deviation.

calculated and finally a standard curves was drawn. A linear correlation coefficient between CT values and log template DNA concentration was determined as $R2 = 0.996 \pm 0.001$. After 30-min incubation, human lysozyme gene evidently bound on the surface of goat spermatozoa about 186,000 copies/spermatozoon in pFLAG-hLY. Also, this gene effectively incorporated into spermatozoon nucleus at 78 copies/spermatozoon. These observations indicated that goat spermatozoa had the ability to captured exogenous DNA spontaneously.

3.2. Spermatozoa motility

After treatment with pFLAG-hLY, goat spermatozoon motility and RPMS showed a significant decrease (Table 1). However, there was no significant difference between incubation control and pre-incubation control, although their motility of both groups showed reduction tendency. The results indicate that incubation with plasmid pFLAG-hLY impaired spermatozoon motility and RPMS.

3.3. Measurement of spermatozoa mitochondrial membrane potentials ($\Delta\Psi m$)

Decreased $\Delta\Psi$m is a sensitive indicator of mitochondrial damage, which was assessed by measuring cellular retention of the fluorescent probe JC-1. The potential across the inner mitochondrial membrane was shown in Fig. 1. In the control group, 34.8% pre-incubation and 30.3% incubation spermatozoa showed normal mean red fluorescent intensity, while this proportion decreased significantly ($P < 0.05$) to 23.74 in the pFLAG-hLY group. The mean green fluorescent intensity in spermatozoa was significantly higher than the control ($74.06 \pm 3.96\%$ vs. pre-incubation control $55.16 \pm 3.16\%$ and incubation control $63.74 \pm 3.55\%$, $P < 0.05$). Further, spermatozoa in plasmid pFLAG-hLY group had a significantly lower ratio of red/green fluorescence intensities of JC-1 (Table 2). These results indicate that incubation with plasmid pFLAG-hLY caused a marked decrease in the mitochondrial membrane potential (MMP) of goat spermatozoa.

3.4. Determination of spermatozoa MDA and ROS levels

The generation of ROS and MDA were induced by 30 min incubation with plasmid pFLAG-hLY. The changes of ROS level in treated spermatozoa were determined by DCF relative fluorescence intensity, as measured by flow cytometry using DCFH-DA as an indicator. There were significant differences in DCF relative fluorescence intensity between the pFLAG-hLY group ($32.81 \pm 2.41\%$) and the pre-incubation control ($15.26 \pm 1.48\%$) and incubation control groups ($16.78 \pm 2.05\%$) (Table 2). ROS production was greater in the pFLAG-hLY group than the other two control groups ($P < 0.05$). Similarly, there was a significantly higher level of MDA in the pFLAG-hLY group (2.18 ± 0.21 nM/10^8 spermatozoa) than in the pre-incubation control (1.43 ± 0.17 nM/10^8 spermatozoa) and incubation control groups (1.54 ± 0.11 nM/10^8 spermatozoa) ($P < 0.05$). In addition, the MDA level moderately or markedly increased throughout the incubation period, though the MDA level in the incubation group was not different from control group (Table 2).

3.5. Determination of mitochondrial cytochrome c expression

The average level of cytochrome *c* release was determined by band densitometry analysis of western blotting (Fig. 2) from three separate experiments. This experiment demonstrated that the fact of spermatozoa taken up pFLAG-hLY significantly induced cytochrome *c* release (11.5 kDa) from the mitochondria into the cytoplasm in the three groups (Fig. 2(a) and (b)). The cytochrome *c* level increased in the cytosolic compartment (204.10 ± 27.67 vs. pre-incubation control 114.90 ± 27.61 and incubation control 142.70 ± 30.91), and this level decreased proportionally in the mitochondria (606.20 ± 29.69 vs. pre-incubation control 707.40 ± 27.18 and incubation control 663.10 ± 32.67). The intensity of cytochrome *c* bands in spermatozoa pellets and supernatants were quantified (Fig. 2(c) and (d)). Comparing with the other two control groups, the levels of cytochrome *c* release were significantly lower in the pellets fractions (mitochondrial) of the

Table 2

Change of MMP, ROS, MDA and Caspase-3 and -9 after goat spermatozoon incubation with pFLAG-hLY.

Parameters	Pre-incubation control	Incubation control	pFLAG-hLY
$\Delta\Psi$m (I_{590}/I_{530}, %)	51.79 ± 2.08^a	58.81 ± 1.76^a	31.44 ± 2.17^b
DCF relative fluorescence (%)	15.26 ± 1.48^a	16.78 ± 2.05^a	32.81 ± 2.41^b
MDA (nM/10^8 spermatozoa)	1.43 ± 0.17^a	1.54 ± 0.11^a	2.18 ± 0.21^b
Caspase-9 (OD_{405})	0.061 ± 0.025^a	0.062 ± 0.019^a	0.083 ± 0.019^b
Caspase-3 (OD_{405})	0.035 ± 0.021^a	0.035 ± 0.025^a	0.086 ± 0.024^b

a, b represent significant differences among groups ($P < 0.05$). Results represent triplicate experiment average and standard deviation.

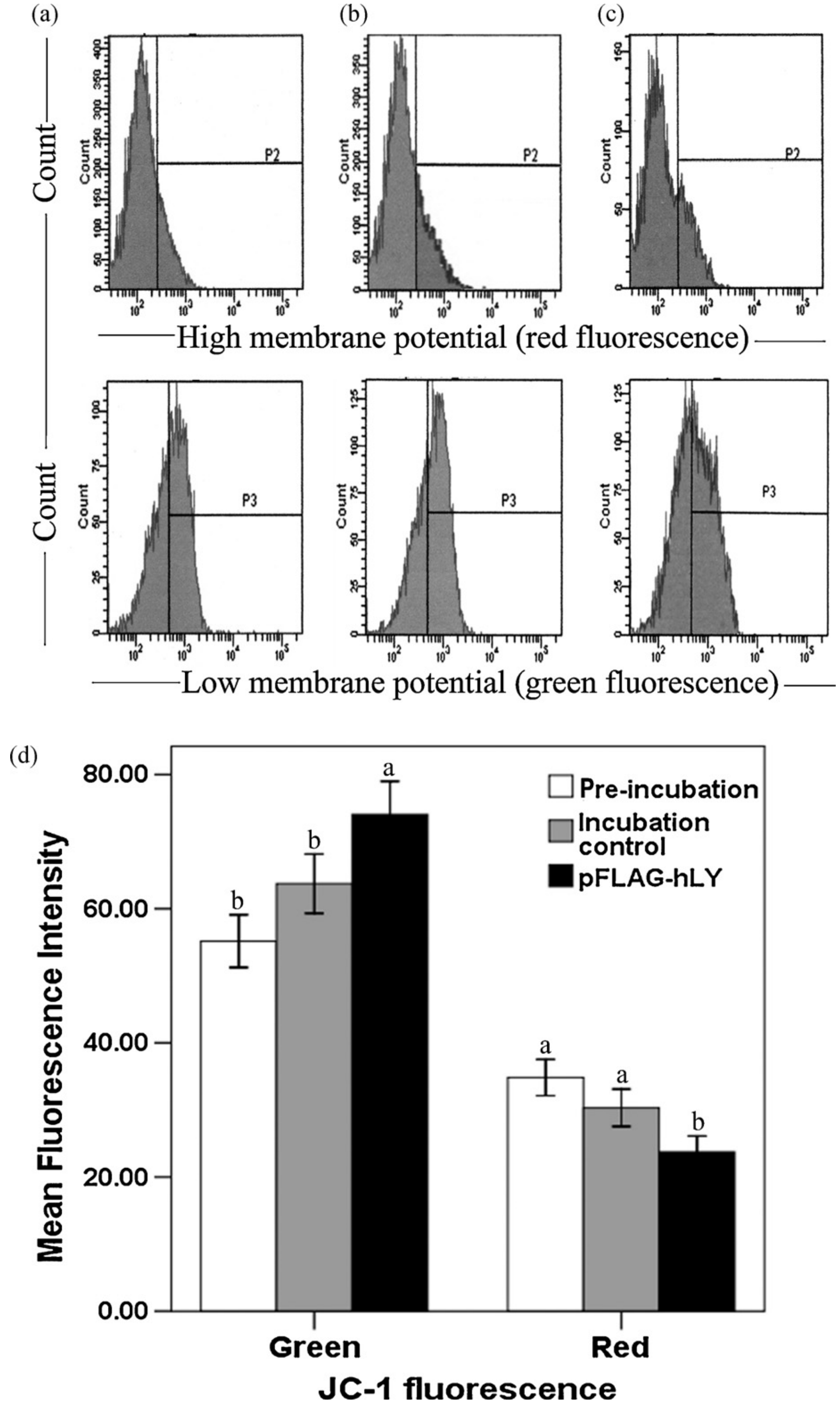

Fig. 1. Flow cytometry study of the mitochondrial membrane potential. (1) A typical picture of flow cytometric analysis of the mitochondrial membrane potential from three groups: the pre-incubation control, the incubation control and pFLAG-hLY. $\Delta\Psi$m was evaluated by flow cytometric analysis of JC-1-stained spermatozoa in figure (a), (b) and (c). Green fluorescence represents the monomeric form of JC-1 with low $\Delta\Psi$m. Red fluorescence represents the mitochondrial aggregate form of JC-1 with high $\Delta\Psi$m. (2) The percentage of red fluorescence intensity and green fluorescence intensity from three groups in figure (d). Different letter a and b represent significant differences among three groups ($P < 0.05$).

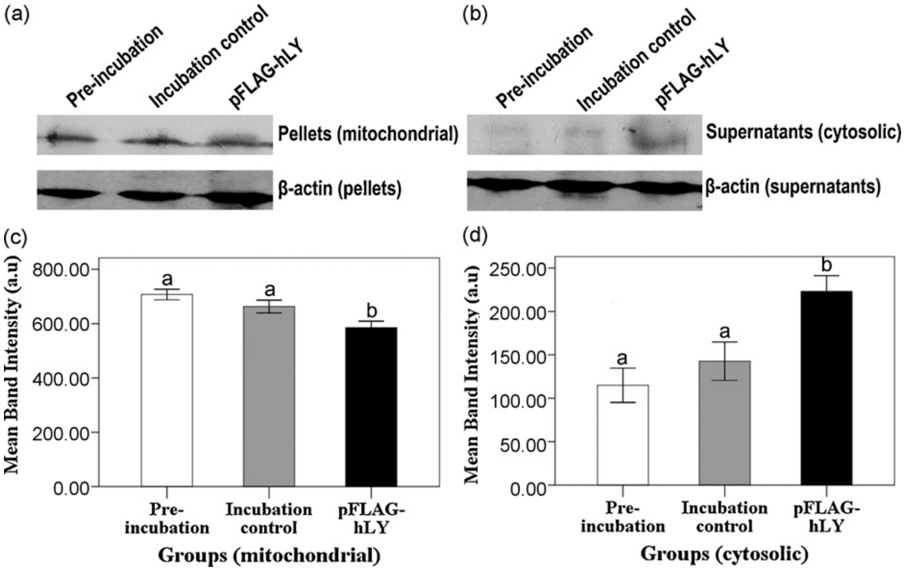

Fig. 2. Western blotting analysis of cytochrome *c* in spermatozoon pellets and supernatants. (1) A representative western blotting of cytochrome *c* expression in spermatozoon pellets (a) and supernatants (b) of three groups; β-actin antibody as loading control and cytochrome *c* was probed using anti-cytochrome *c* antibody. (2) One representative statistical analysis of two similar western blotting from mitochondrial fractions (c) and cytosolic fractions (d) using densitometric analysis software. The densitometric data in cytochrome *c* release was expressed in arbitrary units (a.u.) corresponding bands in spermatozoon pellets from three groups. Different letter a and b represent significant differences among three groups ($P < 0.05$).

pFLAG-hLY group ($P < 0.05$) and there were significantly higher in the supernatants fractions (cytosolic) ($P < 0.05$).

3.6. Colorimetric analysis for the activities of caspase-3 and -9

The enzymatic activities of caspase-3 and -9 were activated in different groups. Caspase-9 is known to be an important trigger in mitochondria and its expression results in spermatozoon dysfunction. Caspase-3 plays a crucial role in mitochondrial oxidative damage. Our study confirmed that 30 min incubation with plasmid pFLAG-hLY induced an increase of caspase-3 and -9 activities (Table 2). The relative levels of caspase-3 and -9 were determined by absorbance at 405 nm. The results showed that the levels of caspase-3 (0.086 ± 0.024) and -9 (0.083 ± 0.019) were higher in treatment group than the controls ($P < 0.05$). No significant differences were observed in enzyme activities between the pre-incubation and incubation control groups.

4. Discussion

The sperm-mediated gene transfer, that uses spermatozoon as a natural vector to introduce new genes into animals, has been considered as a direct, fast and low cost method. However, the effect of this technology is often questionable because of its low efficiency and contradictory reports. Some reports indicated that exogenous DNA invasion may cause change of spermatozoon natural defense system, which led to spermatozoon dysfunction (Kang et al., 2008). Here, our study further analyzed the reason of low efficiency using sperm-mediated vector for transgenic animals by observing sperm mitochondrial function.

The present study showed that after incubation of goat spermatozoa with human lysozyme gene pFLAG-hLY, the plasmid pFLAG-hLY could be bound to the surface of spermatozoon membrane and internalized into spermatozoon nucleus. However, after binding the exogenous pFLAG-hLY plasmid DNA, goat spermatozoa displayed an abnormal motility. At the same time, percentage of rapid progressive motile spermatozoa also changed. This finding was in agreement with previous other animal reports on boars (Gandolfi et al., 1996) and bull (Schellander et al., 1995; Anzar and Buhr, 2006) spermatozoa.

Spermatozoon motility is closely associated with the functional status of spermatozoa mitochondria (Ly et al., 2003) and spermatozoa plasma membrane (Ponzio et al., 2007). Spermatozoa might undergo marked changes in their intracellular and extracellular environment during incubation with exogenous DNA. The most ordinary appearance is reactive oxygen species (ROS) generation. The ROS was responsible for the loss of spermatozoon motility (Bilodeau et al., 2002). Motility is ATP-dependent (Nascimento et al., 2008) and ROS causes a rapid decrease in intracellular ATP concentration (Delamirande and Gagnon, 1992). During incubation of the spermatozoa with pFLAG-hLY, extracellular depletion of ATP in the presence of ROS has a detrimental effect on motility. Intracellular ATP could not neutralize the toxic effect of high ROS formation due to the absent of iron and other transition metal, which led to further decline of spermatozoa motility.

The result of the present study indicated that goat spermatozoa incubation with pFLAG-hLY caused significant decrease in spermatozoon mitochondrial membrane potential ($\Delta\Psi m$). It was also possible that incubation with plasmid pFLAG-hLY had changed culture environment and increased the ROS production, which might interfere spermatozoon mitochondrial function (Guthrie and Welch, 2006). We observed here that significant high level of LPO in treatment group was detected by MDA production (Table 2). This observation reflected that lipid peroxidation (LPO) was an important contributor to mitochondrial dysfunction (Sen et al., 2006) and there was a high positive correlation between percentage of abnormal spermatozoa and membrane LPO (Zheng and Zhang, 1997). Therefore, the potential anomalies of mitochondrial membrane and lipid peroxidation resulted in alterations of mitochondrial function following incubation of plasmid pFLAG-hLY.

Cytochrome *c* release from the mitochondrial inner membrane space has an important role in the induction of oxidative damage. It occurs via mitochondrial permeability transition pore-induced mitochondrial dysfunction (Orrenius et al., 2007). Opening of the permeabilization transition pore complex is thought to mediate release of cytochrome *c* from the mitochondria (Ansari et al., 2006). Mitochondrial permeability transitionresults indisruption of the outer membrane, release of cytochrome *c*, apoptosis inducing factor (AIF) and the activation of caspases (Alksne et al., 2000; Petersen et al., 2000; Gravance et al., 2001; Cordero et al., 2009). Our study showed that the incubation with plasmid pFLAG-hLY caused release of cytochrome *c* via the mitochondrial signaling pathway (Fig. 2). Western blotting analysis revealed that the amount of cytochrome *c* remaining in the mitochondrial pellets displayed significantly reduction. In similar experiments, cytochrome *c* release in the cytosol were markedly increased (Fig. 2). Release of cytochrome *c* is coupled with the increase of activation of caspases-3 and -9 (Table 2). These results strongly supported the fact that the change in mitochondrial membrane potential companied by a partial release of cytochrome *c* from the insoluble fraction to the cytosol. Cytochrome *c* released can interact with the Apaf-1 protein and the inactive form of caspase-9, which leads to activation of caspase-3 (Gravance et al., 2001; Cordero et al., 2009). Therefore, the incubation with plasmid pFLAG-hLY altered spermatozoon mitochondria and induced the expression of cytochrome *c*, the activation of caspase-3 and -9, which led to cytotoxic effect and even spermatozoon death.

In summary, the present study demonstrated that goat spermatozoa could capture human lysozyme plasmid pFLAG-hLY, but the incubation with plasmid pFLAG-hLY resulted in decrease of spermatozoa motility and partial rupture of the spermatozoa mitochondrial membrane, and further promoted the expression of cytochrome *c* and generation of oxidative stress in vitro and finally led to spermatozoon dysfunction. Therefore, a new method needs to be considered to protect DNA-loading spermatozoa from alterations of mitochondrial function and motility during sperm-mediated gene transfer.

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The effect of cryopreservation on goat semen characteristics related to sperm freezability

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ABSTRACT

Seminal quality parameters were used to evaluate the effect of freeze–thawing procedure on goat sperm characteristics, and to relate possible changes in sperm parameters to cryopreservation success. Semen samples ($n = 110$) were frozen with TRIS and milk-based extenders and thawed. Sperm quality parameters (motility, morphology and acrosome) were compared between fresh and frozen–thawed samples. Sperm freezability was judged by classifying the semen samples as "suitable" or "not suitable" according to the sperm quality parameters assessed before and after thawing. Fertility data was obtained after cervical insemination with frozen semen doses. The ejaculates were grouped into two categories according to their fertility results.

In experiment 1, significant differences were found between semen extenders ($P < 0.001$), bucks ($P < 0.05$) and ejaculates within the same male ($P < 0.05$) in terms of sperm quality. There was no seasonal effect ($P > 0.05$) on the majority of the sperm parameters assessed after thawing. Moreover, significant differences ($P < 0.001$) in semen parameters assessed in fresh semen and frozen–thawed samples were found between groups. The effect of the freeze–thawing procedure on sperm quality parameters was also different ($P < 0.05$) between extenders within the same group. The number of sperm quality parameters that had changed after cryopreservation was lower in "suitable" semen samples before and after thawing. In experiment 2, no differences ($P > 0.05$) in semen parameters assessed in fresh semen and frozen–thawed samples were found between groups. The effect of freezing and thawing on sperm quality parameters were different ($P < 0.05$) between extenders within the same group. Only mean beat cross frequency (BCF) values were significantly higher ($P < 0.05$) in TRIS diluted samples that led to successful pregnancies after artificial insemination. In conclusion, CASA-derived motility parameters, together with traditional semen assessment methods, give valuable information on sperm quality before and after freezing. Therefore, the identification of ejaculates as "good" or "bad" based on fresh and post-thaw semen parameters studied in the present experiment were good indicators of goat semen freezability, although the fertilizing capacity of frozen–thawed goat spermatozoa are not revealed by this quality study.

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1. Introduction

The cryopreserved goat semen can be almost indefinitely stored in liquid nitrogen, it facilitates the supply of genetic material, as well as building up gene banks to encourage endangered breeds or valuable individuals (Watson and Holt, 2001), particularly associated with

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artificial insemination (AI) (Leboeuf et al., 2000). This is especially important in the case of Florida male goat, Spanish autochthonous breed under special protection because it is in danger of extinction. However, the biggest obstacle to the exploitation of frozen semen is that the freeze–thawing process of goat sperm generally leads to a decrease in the percentage of motile and viable sperm cells after thawing as a result of damage to membrane integrity and ultrastructure (Watson, 2000).

The evaluation of sperm motility, morphology and acrosomal status is an essential criterion in the evaluation of the quality of a semen sample prior to its use for AI (Salamon and Maxwell, 2000). In this sense, it has been widely demonstrated that cryopreservation leads to a decrease in sperm motility measured objectively by computer-aided methods in the goat (Dorado et al., 2007, 2009) and other animal species (Thurston et al., 2001; Martinez-Pastor et al., 2005). In the case of sperm morphology, it has been demonstrated that a decrease in the number of morphologically normal sperm in ejaculates leads to reduced fertility (Chandler et al., 1988; Gravance et al., 1998). Therefore, the lower fertility of the cryopreserved semen samples may well be a result of a decrease in the number of normal sperm in these samples (Gravance et al., 1997). On the other hand, the presence of an acrosomal cap is important in the fertilization process and has been also highly related with fertility of frozen semen (Lindsay et al., 2005).

The most common methods to evaluate sperm motility is the microscopic observation of semen samples. This subjective estimation has a reduced fertility predictive value because of the high variability observed between individuals and laboratories (Holt et al., 1994; Verstegen et al., 2002). A more objective and precise assessment of sperm motility can be achieved with Computer-Assisted Sperm Analysis (CASA), in which each sperm head trajectory is reconstructed and its kinetics derived in different species (Verstegen et al., 2002; Mortimer and Maxwell, 2004).

Cryopreservation is reported to compromise the fertility of goat spermatozoa based on pregnancy rates from AI (Gacitua and Arav, 2005; Purdy, 2006). There is limited research on motility analysis of goat sperm by the CASA system. Moreover, no attempts have yet been made to associate the motility of goat semen with *in vivo* fertility after AI. The objective of this study was to analyse the effect of the freeze–thawing procedure on goat sperm motility and whether CASA measures can be used as an indicator, together with traditional semen assessment methods, of the sperm freezability and the potential fertility of the cryopreserved sperm.

2. Materials and methods

2.1. Animals and semen collection

Semen was obtained from two mature Florida bucks (2-years-old), located in the south of the Iberian Peninsula (37°53′N–4°46′W). Semen was collected from each animal twice a week, in different and non-consecutive days, using an artificial vagina and estrous females as mounts for the bucks. Ejaculates were equally distributed among males and seasons over a 1-year period.

2.2. Freezing and thawing protocol

After collection, semen samples were diluted with a isothermal glycerol free TRIS-citric acid extender (Biladyl A, Minitüb, Tiefenbach, Germany) at a ratio of 1:9 (semen to Biladyl A, v:v) and centrifuged at 1125 × g for 20 min. The seminal plasma was discarded and the semen pellet was suspended to a final concentration of 250×10^6 rapid progressive spermatozoa/mL (assessed by CASA system) with two different commercial extenders: TRIS-based extender (Triladyl, Minitüb, Tiefenbach, Germany) and skim milk-based extender (Gent, Minitüb, Tiefenbach, Germany) containing both filtered egg yolk (20%, v:v). The diluted sperm suspensions were equilibrated at 5 °C for 5 h and loaded into 0.5 mL straws. They were then frozen in nitrogen vapour for 20 min and transferred to a liquid nitrogen container for storage at −196 °C until used. Thawing was carried out at 24 h after cryopreservation by immersing in a circulating water bath at 39 °C for 30 s.

2.3. Semen evaluation

The volume of each ejaculate was measured in a graduated tube shortly after collection and sperm concentration was determined by manual counting with a Neubauer hemacytometer (Brand, Wertheim, Germany) within 10–20 min after collection.

Aliquots of fresh semen (100 μl) were diluted in DPBS (Dulbecco's phosphate buffered saline, Sigma–Aldrich, Steinheim, Germany) at a sperm concentration of approximately 40×10^6 spermatozoa/mL and used to assess sperm motion. In the in vitro study, an aliquot of diluted semen was incubated at 37 °C for 5 min, before motility evaluation using the computerized system Sperm Class Analyzer (SCA, Microptic SL, Barcelona, Spain). For evaluation, a 10 μl drop of the sample was placed on a pre-warmed Makler chamber (Sefi Medical Instruments Ltd., Haifa, Israel) and a minimum of 200 sperm were counted under a phase contrast microscope (Olympus BH-2, Tokyo, Japan) at 37 °C and 100× magnification. At least 2 drops from each semen sample were evaluated and 3 microscopic fields were filmed for each of the sub-samples.

The semen variables studied were: total motile spermatozoa (MS, %) and rapid progressive motile spermatozoa (RPMS, %); curvilinear velocity (CLV, μm/s); straight line velocity (SLV, μm/s); average path velocity (APV, μm/s); amplitude of lateral head displacement (LHD, μm) and beat cross frequency (BCF, Hz). The main software settings were: frame rates: 25 frames/s, number of frames: 16 frames/object, velocity limit for slow sperm: 30 μm/s, velocity limit for medium sperm: 60 μm/s, minimal linearity (straightness) for progressive fast sperm: 90%, frames min (amplitude of lateral head displacement): 7, and filter for particles: 20%.

The sperm suspension was also used to assess sperm morphology and acrosome integrity. The percentage of spermatozoa with abnormal morphology and intact membrane-acrosome was estimated on Spermac-stained smears (Minitüb, Tiefenbach, Germany). Counts of 200 cells per slide were evaluated using a 100× oil immersion objective with a bright field microscope (Olympus BH-2, Tokyo, Japan).

For post-thaw sperm evaluation, semen straws were also diluted to 40×10^6 spermatozoa/mL with DPBS and again assessed for motility, sperm morphology and acrosome integrity later than 60 min in a 37 °C incubator, as described above.

2.4. *Artificial insemination*

A total of 50 adult females (2–5-year-old) of the Florida breed with proven fertility (had at least one parity) were selected, taking into account their medical and reproductive history and a transrectal ultrasound examination of the reproductive tract. Estrous cycles were synchronised with intra-vaginal sponge impregnated with 45 mg of fluorogestone acetate (Chronogest, Intervet International, Boxmeer, Holland) for 11 days. On day 9, the goats were treated with a single intramuscular injection of eCG (300 IU, Foligon®, Intervet International, Boxmeer, Holland) and 125 μg of cloprostenol (Estrumate®, Schering-Plough Animal Health, Upper Hutt, New Zealand).

A double intra-cervical insemination (using doses from the same ejaculate and extender each time) was carried out 36 and 60 h after sponge removal. Only straws showing a post-thaw rapid progressive motility of 30% or higher were used for inseminations. Each inseminate of 0.5 mL contained on average $8.0 \pm 0.24 \times 10^9$ sperm with rapid progressive motility. Pregnancy diagnosis was performed 25 days after AI by transrectal ultrasonography.

2.5. *Experimental design*

2.5.1. *Experiment 1: effect of cryopreservation on sperm characteristics related to sperm freezability*

One hundred and ten ejaculates from two fertile bucks were frozen–thawed according to the methods described previously. The mean values for semen parameters assessed (motility, morphology and acrosome integrity) were compared between fresh semen and TRIS and milk frozen–thawed extended samples across the entire set of semen samples, including buck, extender and seasons in the model.

Thereafter, sperm freezability was judged by classifying the semen samples as “suitable” or “not suitable” according to standard criteria established by Hidalgo et al. (2007) in accordance with quality assessment of the sperm before and after thawing. Therefore, sperm before freezing were considered as “good quality samples” if they presented an ejaculate volume ≥0.5 mL, sperm concentration $\geq 2 \times 10^9$ sperm/mL and normal morphology ≥70%, and “suitable after thawing” if they displayed ≥30% spermatozoa with rapid progressive motility after an hour in incubation at 37 °C. All samples were frozen and the effect of cryopreservation was investigated in each group. The number of the assessed parameters that had changed after the freeze–thawing procedure was recorded and compared between groups. A multivariate general linear model analyses were also applied to detect possible differences in sperm characteristics between the groups.

2.5.2. *Experiment 2: effect of cryopreservation on sperm characteristics and pregnancy rates*

Twenty three ejaculates were frozen–thawed as described previously and they were used in a field assay by intra-cervical insemination. For AI, only “good quality” semen samples that were also “suitable after thawing” were used. Fifty goats were inseminated with thawed spermatozoa from bucks. A minimum of two goats was inseminated with each semen sample, one for each extender. Ejaculate was considerer to be the experimental unit. First, the ejaculates were grouped into two categories according to whether or not they led to successful pregnancies following AI. Thereafter, the effect of cryopreservation was evaluated in each group and the number of the sperm parameters that had changed after the freeze–thawing procedure was recorded and compared between groups. A multivariate general linear model analyses were also applied to detect possible differences in sperm characteristics between the groups.

2.6. *Statistical analysis*

The results were expressed as mean ± SEM. Data of sperm characteristics were compared using one-way ANOVA, including extender (two extenders), buck (two bucks) and season (four seasons) in the model. Semen samples were classified as “suitable” and “not suitable” according to the sperm quality parameters assessed before and after thawing and to their fertility results. A multivariate analysis of variance (MANOVA) was conducted to check for differences in semen measurements between treatments (fresh semen, TRIS thawed and milk thawed), considering the ejaculate group and treatment as main effects, as well as the interaction between them. When ANOVA revealed a significant effect, values were compared using the Duncan’s multiple range test. A probability of $P < 0.05$ was considered to be statistically significant. The differences in the fertility results between extenders and donors were compared by Fisher’s exact test. Although the tables of means and SEMs show data in the original scale the statistical analyses of sperm variables reported as percentages were performed on arcsin-transformed data using the SAS (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Experiment 1: effect of cryopreservation on sperm characteristics related to sperm freezability*

Motility and acrosome integrity were reduced after freezing and thawing ($P < 0.001$). There was no effect ($P > 0.05$) of cryopreservation on the percentage of morphological abnormalities. Moreover, no significant differences ($P > 0.05$) were found between fresh semen and cryopreserved TRIS samples for LHD (5.49 ± 0.36 versus 6.06 ± 0.56).

Significant differences were found between semen extenders in terms of sperm quality (Table 1). The majority of the post-thaw sperm motility parameters (MS, CLV, SLV, APV, LHD) were significantly higher ($P < 0.001$) in samples processed with TRIS than those diluted in milk extender,

Table 1
Semen parameters of Florida goat spermatozoa after the freeze–thawing process in TRIS and in milk extenders.

Parameter	Extender		Probability
	TRIS	Milk	
Motility parameters			
MS (%)	56.07 ± 1.32	52.25 ± 1.54	0.001
RPMS (%)	33.00 ± 1.02	32.01 ± 1.32	NS
CLV (μm/s)	175.83 ± 11.07	119.24 ± 4.73	0.001
SLV (μm/s)	92.66 ± 1.95	81.23 ± 1.88	0.001
APV (μm/s)	118.71 ± 4.40	93.16 ± 2.68	0.001
LHD (μm)	6.06 ± 0.56	3.57 ± 0.26	0.001
BCF (Hz)	11.00 ± 0.12	11.74 ± 0.09	0.001
Morphological parameters			
ASM (%)	29.89 ± 1.97	27.89 ± 1.92	NS
AMI (%)	47.08 ± 1.43	53.32 ± 1.23	0.001

MS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa; CLV, curvilinear velocity; SLV, straight line velocity; APV, average path velocity; LHD, lateral head displacement; BCF: beat cross frequency; ASM, abnormal sperm morphology; AMI, acrosome membrane integrity. Values are mean ± SEM, $n = 110$.

whereas the opposite was found for BCF and AMI with significantly lower values ($P < 0.001$) in TRIS than in milk extender.

A significant ($P < 0.05$) buck effect was also detected for some of the parameters studied in the frozen–thawed spermatozoa (MS, RPMS, CLV, SLV, APV, LHD, BCF, ASM) (Table 2). A significant ($P < 0.05$) seasonal effect was observed in mean RPMS, CLV, LHD and BCF values after freezing (Table 2). In addition, significant differences ($P < 0.05$) were found between ejaculates within the same male in terms of sperm quality (data not shown).

On the other hand, no differences ($P > 0.05$) were found in the sperm freezability between seasons ("suitable samples for freezing": 11 from spring, 13 from summer, 24 from autumn and 15 from winter; and "suitable samples after thawing": 20 from spring, 28 from summer, 18 from autumn and 20 from winter). On the contrary, significant differences ($P < 0.001$) were found in the sperm freezability between bucks ("suitable samples for freezing": 189 versus 141; and "suitable samples after thawing": 138 versus 192).

Table 3 presents the means of ejaculate volume, sperm concentration and abnormal sperm morphology in fresh semen classified as "good" or "bad" quality before freezing. Significant differences ($P < 0.001$) were found in the percentage of morphological abnormalities between these groups (Table 3).

The MANOVA showed that the effect exerted by the freeze–thawing procedure on sperm parameters was different ($P < 0.001$) depending on the distinction of the semen samples in either "good" or "bad" quality for freezing (Table 4). Likewise, a significant influence ($P < 0.001$) of the treatment on post-thaw sperm parameters was demonstrated. The extender significantly affected ($P < 0.05$) the post-thaw sperm parameters (Table 4). There was an interaction between the ejaculate group and treatment for CLV, APV, LHD and BCF, where motility values of semen samples classified as "good quality for freezing" were statistically higher ($P < 0.01$) than those classified as "not suitable for freezing" in fresh and TRIS diluted semen samples, with the exception of BCF in fresh semen samples (12.03 versus 12.13; $P = 0.6965$). No significant differences were found between groups for those diluted in milk-based extender ($P > 0.05$).

Table 2
Mean ± SEM of the motility parameters and morphological characteristics measured in sperm thawed from two bucks used in the cryopreservation study, both for the overall buck results and for the overall season results.

Parameter	Buck		Season			
	1	2	Spring	Summer	Autumn	Winter
Motility parameters						
MS (%)	48.89 ± 1.17^{a}	60.72 ± 1.54^{b}	93.30 ± 1.13^{a}	92.70 ± 1.09^{a}	90.19 ± 1.48^{a}	89.79 ± 1.30^{a}
RPMS (%)	27.75 ± 0.91^{a}	38.41 ± 1.26^{b}	58.41 ± 2.46^{a}	64.29 ± 2.37^{b}	70.34 ± 1.68^{c}	64.48 ± 1.45^{b}
CLV (μm/s)	133.33 ± 7.47^{a}	165.21 ± 10.42^{b}	246.35 ± 25.40^{b}	157.60 ± 9.49^{a}	198.27 ± 9.53^{a}	187.68 ± 7.05^{a}
SLV (μm/s)	82.25 ± 1.83^{a}	92.84 ± 2.04^{b}	126.68 ± 0.20^{a}	132.88 ± 4.35^{a}	145.17 ± 3.19^{a}	129.49 ± 2.67^{a}
APV (μm/s)	97.86 ± 3.30^{a}	115.98 ± 4.30^{b}	164.18 ± 9.52^{a}	155.78 ± 5.57^{a}	164.47 ± 4.56^{a}	150.84 ± 3.58^{a}
LHD (μm)	4.22 ± 0.38^{a}	5.55 ± 0.53^{b}	8.19 ± 1.28^{b}	5.10 ± 0.48^{a}	4.44 ± 0.53^{a}	4.80 ± 0.34^{a}
BCF (Hz)	11.17 ± 0.11^{a}	11.62 ± 0.96^{b}	12.60 ± 0.20^{b}	12.14 ± 0.24^{ab}	11.80 ± 0.27^{a}	11.85 ± 0.25^{a}
Morphological parameters						
ASM (%)	42.81 ± 2.20^{a}	10.32 ± 1.00^{b}	31.29 ± 4.89^{a}	28.73 ± 4.36^{a}	26.36 ± 4.13^{a}	27.68 ± 2.68^{a}
AMI (%)	89.79 ± 0.62^{a}	91.02 ± 0.69^{a}	93.55 ± 0.66^{a}	87.52 ± 0.87^{a}	88.86 ± 0.70^{a}	92.43 ± 0.91^{a}

MS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa; CLV, curvilinear velocity; SLV, straight line velocity; APV, average path velocity; LHD, lateral head displacement; BCF: beat cross frequency; ASM, abnormal sperm morphology; AMI, acrosome membrane integrity.
Different superscripts (a–c) within the same row and category (buck/season) indicate significant differences ($P < 0.05$).

Table 3
Mean ± SEM of ejaculate volume, sperm concentration and abnormal sperm morphology in fresh semen classified as "good quality for freezing" ($n = 63$) or "not suitable for freezing" ($n = 47$) according to their quality before freezing.

Sperm characteristics	Ejaculates		Probability
	"Good quality for freezing"	"Not suitable for freezing"	
Ejaculate volume (mL)	1.26 ± 0.05	1.12 ± 0.06	NS
Sperm concentration (10^9 sperm/mL)	3.72 ± 0.10	3.66 ± 0.13	NS
Abnormal sperm morphology (%)	13.30 ± 1.05	48.49 ± 2.26	0.001

Table 4
Effect of the cryopreservation procedure on the sperm parameters assessed in “good quality” ($n = 63$) and “not suitable” ($n = 47$) semen samples for freezing.

Ejaculates	Treatment	Motility parameters							Morphological parameters		Wilks’ Λ
		MS (%)	RPMS (%)	CLV (μm/s)	SLV (μm/s)	APV (μm/s)	LHD (μm)	BCF (Hz)	ASM (%)	AMI (%)	
“Good quality for freezing”	Fresh	94.06 ± 0.71^a	67.10 ± 1.25^a	222.66 ± 10.58^a	137.49 ± 2.15^a	166.67 ± 3.94^a	6.31 ± 0.57^a	12.03 ± 0.18^a	13.30 ± 1.05^a	90.74 ± 0.59^a	0.0001; $P < 0.001$
	TRIS	60.24 ± 1.69^b	36.28 ± 1.35^b	202.05 ± 17.35^a	98.28 ± 2.78^b	130.89 ± 6.67^b	7.36 ± 0.88^a	11.35 ± 0.14^b	17.23 ± 1.75^a	48.10 ± 2.03^c	
	Milk	55.89 ± 2.22^b	35.46 ± 1.84^b	120.83 ± 6.35^b	83.85 ± 2.49^c	95.81 ± 3.63^c	3.41 ± 0.30^b	11.85 ± 0.10^a	15.90 ± 1.74^a	54.20 ± 1.72^b	
“Not suitable for freezing”	Fresh	87.84 ± 0.98^a	61.75 ± 1.77^a	182.61 ± 6.41^a	129.82 ± 3.39^a	148.19 ± 3.92^a	4.39 ± 0.30^a	12.13 ± 0.17^a	48.49 ± 2.26^a	89.80 ± 0.73^a	
	TRIS	50.49 ± 1.83^b	28.59 ± 1.34^b	140.69 ± 9.40^b	85.13 ± 2.26^b	102.38 ± 4.11^b	4.31 ± 0.48^a	10.53 ± 0.18^c	46.85 ± 2.25^a	45.74 ± 1.98^c	
	Milk	47.37 ± 1.85^b	27.38 ± 1.65^b	117.11 ± 7.15^c	77.72 ± 2.82^b	89.60 ± 3.96^c	3.78 ± 0.46^a	11.60 ± 0.15^b	43.73 ± 2.30^a	52.15 ± 1.73^b	

MS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa; CLV, curvilinear velocity; SLV, straight line velocity; APV, average path velocity; LHD, lateral head displacement; BCF: beat cross frequency; ASM, abnormal sperm morphology; AMI, acrosome membrane integrity.

Values are mean ± SEM. Differences between fresh semen, TRIS and milk extenders were described by letters (a–c). Different letters between treatments for the “good quality for freezing” and “not suitable for freezing” groups indicate significant differences ($P < 0.05$). The MANOVA Wilks’ Λ tested with F value.

The number of sperm parameters that were changed by cryopreservation varied between “good” and “bad” quality samples before freezing (Table 4). Therefore, three parameters (CLV, LHD, ASM) from TRIS samples classified as “good quality before freezing” were not changed by cryopreservation and two (LHD, ASM) was not changed in “bad quality samples before freezing”. In addition, two parameters (BCF, ASM) from milk samples classified as “good quality before freezing” was not changed by cryopreservation and two (LHD, ASM) not changed in “bad quality before freezing”.

Table 5 presents the means of ejaculate volume, sperm concentration and abnormal sperm morphology in fresh semen classified as “good” or “bad” quality after thawing. As in the case of groups formed in accordance with sperm quality parameters assessed before freezing, significant differences ($P < 0.001$) were found in the percentage of morphological abnormalities between groups (Table 5).

The effect exerted by the freeze–thawing procedure on sperm quality parameters were different ($P < 0.001$) and the semen samples were classified as “suitable” or “not suitable” after thawing (Table 6). A significant influence ($P < 0.001$) of the treatment on post-thaw sperm parameters was also demonstrated. Similarly, the extender significantly affected ($P < 0.05$) the post-thaw sperm parameters (Table 6). The interaction ($P < 0.001$) between the ejaculate group and treatment for MS, RPMS, SLV and APV, where motility values of semen samples classified as “suitable after thawing” were statistically higher than those classified as “not suitable after thawing” in TRIS and milk diluted semen samples. No significant differences were found between groups for fresh semen samples ($P > 0.05$) with the exception of MS (94.71 versus 89.03; $P < 0.001$).

The number of sperm parameters that were changed by cryopreservation varied between “suitable” and “not suitable” samples after thawing (Table 6). Three parameters (CLV, LHD, ASM) from TRIS samples classified as “suitable after thawing” were not changed by cryopreservation and two (LHD, ASM) were not changed in “not suitable after thawing”. Furthermore, three parameters (LHD, BCF, ASM) from milk samples classified as “suitable after thawing” were not changed by cryopreservation and one (ASM) was not changed in “not suitable after thawing”. Therefore, the number of sperm parameters that had not changed after cryopreservation increased in “suitable semen samples after thawing”.

3.2. *Experiment 2: effect of cryopreservation on sperm characteristics and pregnancy rates*

The overall fertility obtained after cervical insemination was 38% (19/50). Pregnancy rates for TRIS (35.7%) were minimally lower than those obtained for milk (40.9%), although no significant differences were found between the two extenders ($P = 0.466$) and donors (36.4% versus 39.3%; $P = 0.534$).

Table 7 presents the means of ejaculate volume, sperm concentration and abnormal sperm morphology in fresh semen classified as “successful” or “not successful” pregnancy after artificial inseminations. No significant differences ($P > 0.05$) were found between groups for these parameters (Table 7).

Table 5
Mean ± SEM of ejaculate volume, sperm concentration and abnormal sperm morphology in fresh semen classified as "suitable after thawing" ($n = 46$) or "not suitable after thawing" ($n = 64$) according to their quality after thawing.

Sperm characteristics	Ejaculates		Probability
	"Suitable after thawing"	"Not suitable after thawing"	
Ejaculate volume (mL)	1.13 ± 0.07	1.26 ± 0.42	NS
Sperm concentration (10^9 sperm/mL)	3.70 ± 0.12	3.69 ± 0.11	NS
Abnormal sperm morphology (%)	18.52 ± 2.32	35.40 ± 2.72	0.001

The effect exerted by the freeze–thawing procedure on sperm quality parameters was not different ($P > 0.05$) between semen samples classified according to whether or not they led to successful pregnancies following AI (Table 8). However, mean BCF values were statistically higher (11.98 versus 11.04; $P = 0.0151$) in TRIS diluted samples that led to successful pregnancies than those that did not following AI. A significant ($P < 0.01$) influence of the treatment on post-thaw sperm parameters has also been demonstrated by MANOVA. Therefore, mean values of CLV, SLV, APV and LHD were statistically higher ($P < 0.05$) in semen samples diluted with TRIS compared to those diluted with milk extender (Table 8). There was no interaction ($P > 0.05$) between the ejaculate group and treatment on the post-thaw sperm parameters.

The number of sperm parameters that had changed after the freeze–thawing procedure varied between the semen samples that led to successful pregnancies following the AI and those that did not. Five parameters (CLV, APV, LHD, BCF, ASM) from semen samples with TRIS and four parameters (CLV, LHD, BCF, ASM) from milk straws followed by pregnant goats were not changed after cryopreservation (Table 8). In addition, four parameters (CLV, LHD, BCF, ASM) from TRIS and two parameters (BCF, ASM) from milk were not affected by the freeze–thawing procedure in those samples that did not lead to successful pregnancies after AI.

4. Discussion

In the present study, cryopreservation provoked a reduction in the motility parameters and acrosome integrity of the overall frozen samples ("suitable" and "not suitable"); however, values obtained in "suitable" semen samples are comparable to those from other studies (Ritar and Ball, 1993; Aboagla and Terada, 2004; Purdy, 2006).

The extender used significantly influenced the response of the overall sperm motility to freeze–thawing. The effect of different extenders and cryoprotectants on sperm motility has been widely documented in other animal species (Thurston et al., 2001; Garde et al., 2003; Martinez-Pastor et al., 2005). In our conditions, sperm motility parameters were lower in both extenders than those of fresh semen, although with the milk extender the reduction of the motility parameters was even greater than with TRIS. This difference between extenders might suggest that the TRIS extender provides greater protection of the parts of the spermatozoa involved in locomotion, since the reduction in motility was less marked. On the other hand, in this study, the percentage of sperm with normal acrosomes was significantly reduced after freezing and thawing. In agreement with previous findings of Gil et al. (2000), the milk extender provided more effective preservation of the acrosomal membrane after thawing compared to TRIS extender. Similarly, slightly higher pregnancy rates were observed in the milk extender frozen samples, although these differences were not significant.

The sperm characteristics were not modified throughout the experimental period, showing reasonably constant mean values for 1 year after freezing. These findings are in agreement with other published data reporting no variation of the post-thaw semen motility throughout the experimental period (Cabrera et al., 2005). On the other hand, the results of our study detected differences between bucks in the ability of semen to remain motile after freezing. Inter-male variability in sperm freezability has been recognized as a source of variation during freeze–thawing (Watson, 1995; Holt, 2000) and it seems necessary to asses the semen quality after freezing–thawing to define the donors bucks as "good" or "bad" freezers (Leboeuf et al., 2000).

The detrimental effects of cryopreservation on sperm quality and viability had been widely demonstrated. In this study, in order to determine whether changes in sperm characteristics could also be used as a possible indicator of the detrimental effect of cryopreservation, the effect of freeze–thawing process was examined in semen samples classified according to whether the semen quality was assessed as "suitable" or "not suitable" before freezing and after thawing. Out of the 110 ejaculates included, 46 were identified as "good" and 64 as "bad", according with their freezability. The cryodamage rate observed in our experiment may not necessarily be a result of the cryopreservation conditions applied, which might also depend on individual genetic variation. Variability in semen freezability between animals or ejaculates is a well known event of semen cryopreservation (Watson, 1995) and there appear to be genetic bases for variations in post-thaw semen quality between individuals, which will make the semen of certain males consistently freeze badly, irrespective of the conditions provided (Thurston et al., 2002). Although individual variation was observed in post-thaw sperm quality and in the sperm freezability, we cannot conclude that there was animal effect on sperm parameters assessed after freezing due to the fact that only two males were used in this study. Therefore, further studies should be conducted to determine the influence of males and ejaculates on sperm freezability of Florida goat in different seasons and their interaction.

It is also interesting to observe that the number of sperm parameters that had not changed after cryopreservation was increased in "suitable" samples after thawing. Indeed, some of CASA motion parameters (MS, RPMS,

Table 6
Effect of the cryopreservation procedure on the sperm parameters assessed in samples classified as “suitable” ($n = 46$) or “not suitable” ($n = 64$) after thawing.

Ejaculates	Treatment	Motility parameters							Morphological parameters		Wilks’ Λ
		MS (%)	RPMS (%)	CLV (μm/s)	SLV (μm/s)	APV (μm/s)	LHD (μm)	BCF (Hz)	ASM (%)	AMI (%)	
“Suitable after thawing”	Fresh	94.71 ± 0.70^a	65.64 ± 1.79^a	220.70 ± 13.29^a	136.03 ± 3.00^a	164.38 ± 5.08^a	6.27 ± 0.70^{ab}	12.12 ± 0.26^a	18.52 ± 2.32^a	91.58 ± 0.64^a	0.0001; $P < 0.001$
	TRIS	66.71 ± 1.20^b	40.86 ± 1.00^b	213.04 ± 19.02^a	102.47 ± 2.55^b	137.52 ± 6.99^b	7.84 ± 0.98^a	11.35 ± 0.16^b	20.51 ± 2.64^a	51.42 ± 2.10^c	
	Milk	65.72 ± 1.45^b	43.85 ± 1.24^b	143.71 ± 8.46^b	93.96 ± 2.10^c	110.56 ± 3.93^c	4.25 ± 0.44^b	11.95 ± 0.10^a	17.73 ± 2.31^a	56.40 ± 1.63^b	
“Not suitable after thawing”	Fresh	89.03 ± 0.89^a	64.21 ± 1.31^a	194.66 ± 6.79^a	132.91 ± 2.52^a	154.74 ± 3.42^a	4.93 ± 0.35^a	12.03 ± 0.12^a	35.40 ± 2.72^a	89.45 ± 0.62^a	
	TRIS	48.43 ± 1.49^b	27.34 ± 1.18^b	149.08 ± 12.30^b	85.61 ± 2.48^b	105.19 ± 5.05^b	4.78 ± 0.62^a	10.74 ± 0.16^c	36.62 ± 2.50^a	44.17 ± 1.86^c	
	Milk	42.57 ± 1.57^c	23.50 ± 1.28^c	101.66 ± 4.24^c	72.08 ± 2.25^c	80.65 ± 2.75^c	3.07 ± 0.30^b	11.60 ± 0.12^b	35.01 ± 2.49^a	51.18 ± 1.71^b	

MS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa; CLV, curvilinear velocity; SLV, straight line velocity; APV, average path velocity; LHD, lateral head displacement; BCF: beat cross frequency; ASM, abnormal sperm morphology; AMI, acrosome membrane integrity.
Values are mean ± SEM. Differences between fresh semen, TRIS and milk extenders were described by letters (a–c). Different letters between treatments for the “suitable after thawing” and “not suitable after thawing” groups indicate significant differences ($P < 0.05$). The MANOVA Wilks’ Λ tested with F value.

Table 7
Mean ± SEM of ejaculate volume, sperm concentration and percentages of sperm motility, abnormal sperm morphology and acrosome integrity in fresh semen classified as “successful pregnancies” ($n = 19$) or “not successful pregnancies” ($n = 31$) according to their fertility results.

Sperm characteristics	Ejaculates		Probability
	“Successful pregnancies”	“Not successful pregnancies”	
Ejaculate volume (mL)	1.29 ± 0.06	1.29 ± 0.07	NS
Sperm concentration (10^9 sperm/mL)	4.01 ± 0.16	3.87 ± 0.16	NS
Abnormal sperm morphology (%)	17.79 ± 1.75	17.08 ± 1.80	NS

SLV, APV) were significantly higher in both TRIS and milk diluted samples classified as “suitable after thawing” than those classified as “not suitable after thawing”. It has been previously shown that in higher quality samples the sperm cryodamage was lower, and consequently the freeze–thawing effect on the sperm quality was also lower, indicating better freezability. Therefore, it might be assumed that the identification of ejaculates as “good” or “bad” based on post-thaw semen parameters studied in the present experiment were good indicators of goat semen freezability.

Recent studies on humans (MacLeod and Irvine, 1995), bulls (Amann, 1989), stallions (Vidament et al., 2000b), boars (Holt et al., 1997), rabbits (Farrell et al., 1993) and rats (Moore and Akhondi, 1996) have examined the relationship between several CASA motility parameters and the *in vivo* fertility. Others have failed to determine an association between motion parameters of cryopreserved samples and fertility after AI in bull (Bailey et al., 1994), sheep (Sanchez-Partida et al., 1999) and stallion (Nie et al., 2002). In any case, it is also well known that the positive relationship between single semen quality characteristics such as motility and fertility is either of low significance or measurable in some sires but not in others, as reported by Gil et al. (2005).

This study demonstrated the relationship between CASA motility parameters and the fertility of cryopreserved caprine semen. The fertility results obtained in the field trial were 38%, which is in the range reported in literature. Since good quality semen samples are not always related to high pregnancy rates, bad quality samples are rarely related to high fertility. Hence, in this experiment, semen samples that were “suitable” before and after cryopreservation were used with the aim of verifying if there are subtle changes in the sperm quality parameters that might differentiate between semen samples with adequate semen quality but with different pregnancy rates. For this purpose, the effect of freeze–thawing procedure was compared between the sperm samples that led to successful pregnancies and those that did not following AI.

The results indicate that for the TRIS frozen–thawed extended samples, only mean BCF values were significantly higher in semen samples leading to successful pregnancies than in those that did not achieve this result after AI. Moreover, when a comparison of the number

Table 8
Effect of the cryopreservation procedure on the sperm parameters assessed in samples leading to successful ($n = 19$) or not successful pregnancies ($n = 31$) after artificial inseminations.

Ejaculates	Treatment	Motility parameters							Morphological parameters		Wilks' Λ
		MS (%)	RPMS (%)	CLV (μm/s)	SLV (μm/s)	APV (μm/s)	LHD (μm)	BCF (Hz)	ASM (%)	AMI (%)	
"Successful Pregnancies"	Fresh	91.72 ± 2.62^a	65.32 ± 2.41^a	218.27 ± 9.47^{ab}	135.66 ± 3.27^a	163.90 ± 4.62^a	6.34 ± 0.49^{ab}	12.01 ± 0.44^a	17.79 ± 1.75^a	90.93 ± 0.93^a	0.5531; NS
	TRIS	65.57 ± 1.76^b	39.43 ± 2.23^b	275.42 ± 55.25^a	114.02 ± 4.51^b	163.84 ± 4.51^a	10.58 ± 2.78^a	11.98 ± 0.26^a	22.94 ± 5.01^a	59.94 ± 3.80^b	
	Milk	61.97 ± 2.10^b	39.91 ± 2.01^b	129.38 ± 15.18^b	87.42 ± 5.28^c	99.95 ± 8.11^b	3.38 ± 0.66^b	12.14 ± 0.11^a	25.01 ± 5.58^a	54.58 ± 2.36^b	
"Not successful pregnancies"	Fresh	93.18 ± 1.74^a	67.09 ± 1.75^a	215.69 ± 9.80^a	139.83 ± 2.78^a	166.41 ± 4.32^a	5.98 ± 0.52^a	11.66 ± 0.42^a	17.08 ± 1.80^a	90.41 ± 0.96^a	
	TRIS	63.18 ± 1.85^b	39.89 ± 1.44^b	200.98 ± 26.10^a	105.71 ± 3.50^b	137.05 ± 10.44^b	6.70 ± 1.36^a	11.04 ± 0.20^a	23.03 ± 4.45^a	56.23 ± 3.06^b	
	Milk	64.85 ± 2.85^b	44.37 ± 2.62^b	122.43 ± 6.46^b	92.60 ± 4.20^c	103.13 ± 4.88^c	3.14 ± 0.28^b	12.05 ± 0.16^a	16.08 ± 2.46^a	50.32 ± 3.93^b	

MS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa; CLV, curvilinear velocity; SLV, straight line velocity; APV, average path velocity; LHD, lateral head displacement; BCF: beat cross frequency; ASM, abnormal sperm morphology; AMI, acrosome membrane integrity.
Values are mean ± SEM. Different letters between treatments for the "successful pregnancies" or "not successful pregnancies" groups indicate significant differences ($P < 0.05$). The MANOVA Wilks' Λ tested with F value.

of sperm parameters that had not changed after cryopreservation between ejaculates group was made, APV did not change after cryopreservation in semen samples leading to successful pregnancies after AI. Many CASA motion parameters (LHD, BCF, LIN, APV, SLV) have been associated with fertility in bulls (Farrell et al., 1998). In this study, motility assessed by the SCA system give valuable information on post-thaw sperm quality, but the fertilizing capacity of frozen–thawed goat spermatozoa are not revealed by this quality study. The high quality of frozen semen doses used in the field trial might be responsible for these results, resulting in higher final numbers of suitable spermatozoa in the insemination dose. These results might also be due to the fact that the numbers of spermatozoa per dose used in our experiments exceeds the number of sperm needed to obtain the highest fertility achievable for most males. Therefore, the fertilizing potential of individual sperm in most semen samples could be masked by the excess sperm inseminated, as described in bulls (Shannon and Vishwanath, 1995; den Daas et al., 1998; Farrell et al., 1998), rabbits (Farrell et al., 1993) and boars (Tardif et al., 1999). For all these reasons, semen motility analysis might allow us to allocate of insemination doses into "good" or "bad" based on post-thaw motility and exclude those samples or even males from an AI program. Nevertheless, this analysis could not predict fertilizing potential of high quality insemination doses. To avoid this limitation, further studies are needed to assess the importance that motile sperm subpopulations given by CASA analysis could have on the fertilizing potential of caprine semen frozen, when high quality insemination doses are used in field trial.

In conclusion, the effect exerted by cryopreservation was not equal for the extenders, males and seasons, although no differences were found in the sperm freezability between seasons. CASA-derived motility parameters, together with traditional semen assessment methods, give valuable information on sperm quality before and after freezing. So, the number of sperm parameters that had changed after cryopreservation was lower in semen samples classified as "suitable" before and after thawing, which showed greater resistance to the freeze–thawing process. This suggest that the identification of ejaculates as "good" or "bad" based on fresh and post-thaw semen parameters studied in the present experiment were good indicators of goat semen freezability. However, their use as a predictive tool for the potential fertility of cryopreserved sperm of the Florida buck has not been fully tested in this work.

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Endocrine alterations around the time of abortion in mares impregnated with donkey or horse semen

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ABSTRACT

The objective of this study was to monitor and compare the concentrations of equine chorionic gonadotropin (eCG), progesterone and estrone sulphate during normal and failed pregnancies of mares impregnated with donkey or horse semen, relating their individual endocrine profiles to the time of pregnancy loss, and to the histopathologic findings in the aborted fetuses and placenta. Mares ($n = 54$) were used, 32 of them impregnated with donkey semen and 22 impregnated with horse semen. Blood samples were taken twice a week from Day 35 to 120 of pregnancy. Ultrasonographic observations of the fetus were carried out twice a week. The incidence of abortion in mares impregnated with donkey semen (30%) was greater ($P < 0.05$) than the 5% observed in mares impregnated with horse semen. From Week 8 to the end of the sampling period, the mean progesterone concentrations of mares with normal mule pregnancies were less ($P < 0.05$) than those of mares with normal pregnancies with equine fetuses. The concentrations of eCG were less ($P < 0.05$) in mule pregnancies from Week 6. Estrone sulphate concentrations were only different ($P < 0.05$) between types of pregnancy on Weeks 13 and 14, being in this case greater with the mule pregnancies.

Most of the abortions of mule fetuses were associated with lesser progesterone concentrations than the average for mares with successful mule pregnancies. Four of the abortions of mule fetuses and the only abortion of horse fetus occurred in mares with lesser progesterone and very low eCG concentrations, and were classified as caused by luteal impairment secondary to eCG deficiency; estrone sulphate concentrations were less than normal or absent before these abortions. Two mares aborted after several weeks of low progesterone concentrations in the presence of eCG concentrations that were normal for mule pregnancies, suggesting primary luteal deficiency. In three mares carrying a mule fetus, the concentrations of progesterone and estrone sulphate decreased abruptly immediately before fetal death, suggesting luteolysis due to active prostaglandin F2 alpha (PGF2α) secretion. It is concluded that the greater incidence of abortion in mares impregnated by donkeys is associated with different kinds of luteal malfunction. Deficiency of eCG may be a primary cause of many of these cases, either by failing to stimulate enough luteal progesterone secretion and/or by failing to protect the corpora lutea (CL) of pregnancy from endogenous PGF2α secretion.

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1. Introduction

During the normal pregnancy of a mare impregnated by a horse, the endometrial cups secrete equine chorionic gonadotropin (eCG) from Day 35 to 40 and Day 120 to 150

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of gestation (Allen, 2001a; Boeta and Zarco, 2005). After that time, the endometrial cups are rejected by the mare's immune system (Allen, 2001a). Working in concert with FSH, eCG induces the formation of supplementary CL during pregnancy (Allen, 2001a). In addition, eCG stimulates progesterone production by the primary and the supplementary CL (Bergfelt et al., 1989; Allen, 2001a).

In pregnant mares eCG is an important regulator of luteal steroidogenesis. Thus, from Day 40 to 150 of pregnancy the CL secrete increased amounts of progesterone in mares that have been impregnated by a horse (Daels et al., 1998). When a mare is cross-bred with a donkey to produce a mule, however, the endometrial cups derived from chorionic cells of the mule embryo are promptly rejected and eCG concentrations do not increase, or increase only slightly from Day 40 to 60, decreasing to undetectable concentrations between the 7th and 11th week of gestation (Allen, 1982, 2001a; Boeta and Zarco, 2005). This lack of gonadotropic support results in the formation of few supplementary luteal structures (Allen, 1982; Allen et al., 1987) and in progesterone concentrations well below those found during pregnancies of mares served by stallions (Boeta and Zarco, 2005).

The importance of eCG for pregnancy maintenance in the mare is not clear. Low fertility reported for mares used to produce mules (Jordao et al., 1954) could in part be due to a greater incidence of abortions preceded by low eCG concentrations and inadequate luteal function (Boeta and Zarco, 2005). However, many mares carry their mule foals successfully to term in spite of very low and even undetectable eCG concentrations throughout gestation (Boeta and Zarco, 2005).

To our knowledge, there are no reports of estrone sulphate profiles during pregnancies of mares carrying mule fetuses. Monitoring the concentrations of this hormone (Terqui and Palmer, 1979; Madej et al., 1987) during successful and failed pregnancies of these mares can provide additional information on the effects of eCG deficiency on this type of gestation, specifically with regard to the function of the feto-placental unit. Thus, the objective of this study was to monitor and compare eCG, progesterone and estrone sulphate concentrations during normal and failed pregnancies of mares impregnated by donkeys and by horses, relating their individual endocrine profiles to the time of pregnancy loss and to the histopathologic findings in the aborted fetuses and placenta.

2. Materials and methods

Adult healthy mares ($n = 54$) were used: 32 impregnated with donkey semen and 22 impregnated with horse semen. Blood samples for the determination of serum concentrations of progesterone, eCG and estrone sulphate were taken by jugular venepuncture from each mare twice a week from Day 35 to 120 of pregnancy. Progesterone concentrations were determined by a solid-phase radioimmunoassay (Pulido et al., 1991), with intra- and inter-assay variation coefficients of 4.1% and 7.3%, respectively. Estrone sulphate concentrations were determined by solid-phase radioimmunoassay (Estrone Sulfate RIA test, Diagnostic Products Corporation, Los Angeles, CA, USA) with intra and inter-assay variation coefficients of 4.2% and 5.3%. Concentrations of eCG were measured by an enzyme-immunoassay (PMSG-ELISA, DRG Instruments, Marburg, Germany) with intra- and inter-assay variation coefficients of 7.4% and 10.0%, respectively. Ultrasonographic observations of the fetus were conducted twice a week. Fetal death was considered to have occurred when no cardiac beat or fetal movements were found during an ultrasonographic examination, or when the fetus was expelled during the interval between examinations (Boeta and Zarco, 2005).

Initially, the weekly mean concentrations of progesterone, eCG and estrone sulphate of the mares with normal pregnancies of each type (mares impregnated by donkey or horse) were compared using analysis of variance for repeated measures. Tukey's multiple comparisons were used to compare differences between groups in different weeks. Thereafter, the hormonal profiles of each of the aborting mares were compared against the means for the normal pregnancies of the same type, and the mares that aborted were classified according to their probable cause of abortion (primary luteal deficiency, luteal impairment secondary to eCG deficiency, active luteolysis) as described by Boeta and Zarco (2005). Then, the weekly mean concentrations of progesterone, eCG and estrone sulphate for each class of abortion of mule fetuses were compared against those for normal mule pregnancies using an analysis of variance for repeated measures, followed by Tukey's multiple comparisons. Necropsies and histopathology studies of all aborted fetuses and placenta were conducted.

3. Results

The general incidence of embryonic mortality (before Day 40 of pregnancy) was 7.4%, resulting from 2/32 mares that lost their mule embryos on Days 21 and 32, and 2/22 mares that lost their equine embryos on Days 38 and 40. The difference between types of gestation was not significant ($P > 0.05$).

The incidence of abortion (after Day 40) in mares impregnated with donkey semen was 30% (9/30), occurring between Days 56 and 98. In mares impregnated with horse semen the incidence of abortion was 5% (1/20), occurring at 103 days of gestation. There was difference ($P < 0.05$) in the incidence of abortion between types of gestation.

There were effects ($P < 0.01$) of type of pregnancy (mule or horse fetus), week of pregnancy and the interaction between type of pregnancy and week on the mean concentrations of progesterone, eCG and estrone sulphate. As depicted in Fig. 1, from Week 8 to the end of the sampling period the mean progesterone concentrations of mares with normal mule pregnancies were less ($P < 0.05$) than those of mares with normal pregnancies with horse fetuses. The concentrations of eCG were less ($P < 0.05$) in mule pregnancies from Week 6. Estrone sulphate concentrations were only different ($P < 0.05$) between types of pregnancy on Weeks 13 and 14, being in this case greater in the mule pregnancies.

After the evaluation of the hormone profiles of individual mares as related to time of pregnancy loss, the mares that aborted mule fetuses were classified as mares with secondary luteal deficiency ($n = 4$), primary luteal defi-

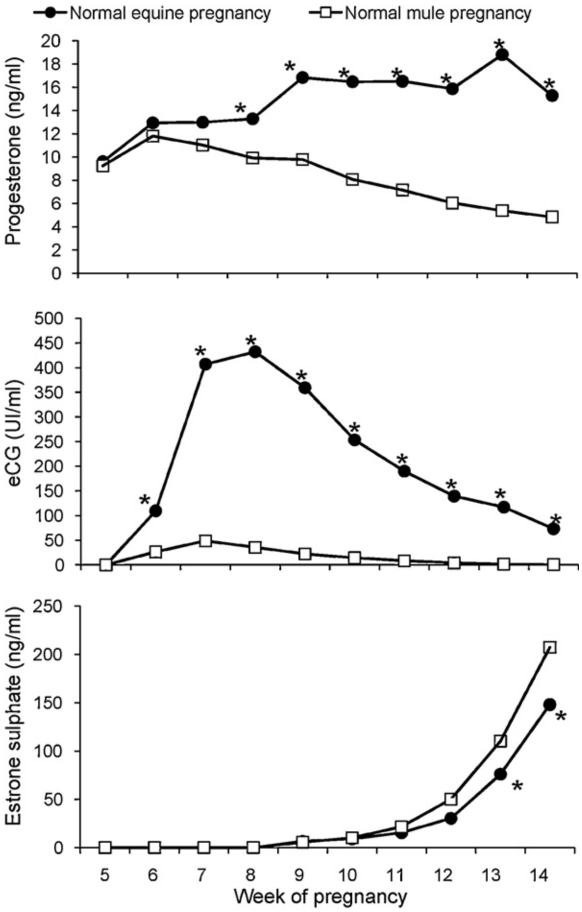

Fig. 1. Weekly mean concentrations of progesterone, eCG and estrone sulphate during pregnancy of mares carrying equine or mule fetuses to term (normal pregnancies). An asterisk near a point indicates differences ($P < 0.05$) between groups on that week.

ciency ($n = 2$) and active luteolysis ($n = 3$). The only abortion of a horse fetus was associated with secondary luteal deficiency.

As depicted in Fig. 2, in the mares with secondary luteal deficiency both progesterone and eCG concentrations were, for several weeks before the occurrence of abortion, less ($P < 0.05$) than those found during normal mule pregnancies. Estrone sulphate concentrations in those mares never increased. No alterations were found at histopathology in three of these abortions. Fig. 3 depicts the precise relation between the endocrine changes and the time of abortion in one of these mares. The fourth case was associated with bacterial placentitis in a mare (Fig. 4) that had normal progesterone concentrations during the first 8 weeks of pregnancy, and even formed a supplementary CL on Week 8 in spite of a total absence of eCG. At Week 8 she was bitten in the vulva by another mare and developed an infection; progesterone concentrations decreased during the following week and remained low until the time of abortion, and estrone sulphate failed to increase.

In two mares with primary luteal deficiency the abortions were preceded by several weeks with lesser ($P < 0.05$) progesterone concentrations, even though their eCG concentrations were not different ($P > 0.05$) than those of the mares with normal mule pregnancies (Fig. 2). Estrone sulphate never increased in these two mares and no alterations were found at histopathology. Fig. 5 depicts the precise relation between endocrine changes and the time of abortion in one of these mares.

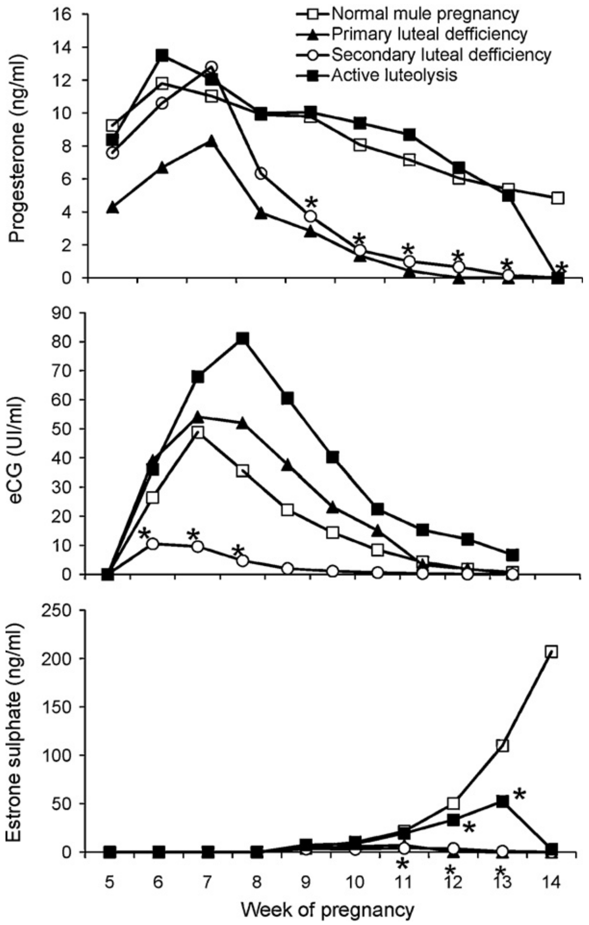

Fig. 2. Weekly mean concentrations of progesterone, eCG and estrone sulphate during normal pregnancies with mule fetuses or during aborted pregnancies associated with different endocrine alterations. An asterisk near a point indicates a difference ($P < 0.05$) on that week with respect to the normal pregnancies. The asterisks on the progesterone graph are valid for both primary and secondary luteal deficiency.

In the other three mares that aborted a mule fetus the concentrations of progesterone and estrone sulphate were normal during most of pregnancy, but decreased abruptly immediately before fetal death (Fig. 2), suggesting active luteolysis due to PGF2α secretion (Daels et al., 1989, 1991). The concentrations of eCG in these mares tended to be greater than those of normal pregnancies, but the differences were not significant ($P > 0.05$). No alterations were found at histopathology in the placenta or fetus of two of the mares in this class; Fig. 6 depicts the precise relation between endocrine changes and the time of abortion in one of these mares. Focal necrotic hepatitis was found in the third fetus, which was aborted on Week 14.

The only abortion of a horse fetus (Fig. 7) occurred on a mare that always had progesterone and eCG con-

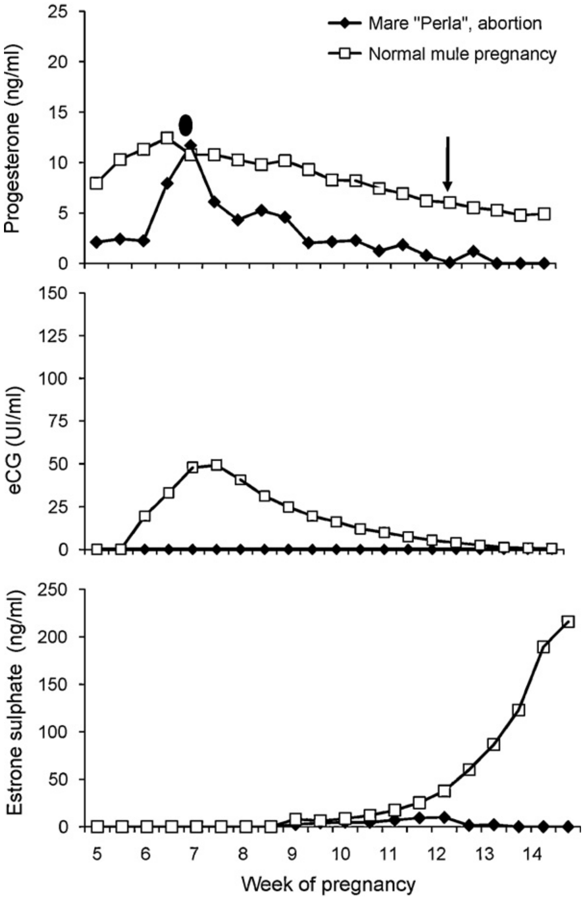

Fig. 3. Progesterone, eCG and estrone sulphate concentrations in a mare that aborted a mule fetus after a period of luteal deficiency secondary to the absence of eCG secretion. The arrow indicates the time of fetal death. The black oval at Week 7 indicates the formation of a supplementary corpus luteum.

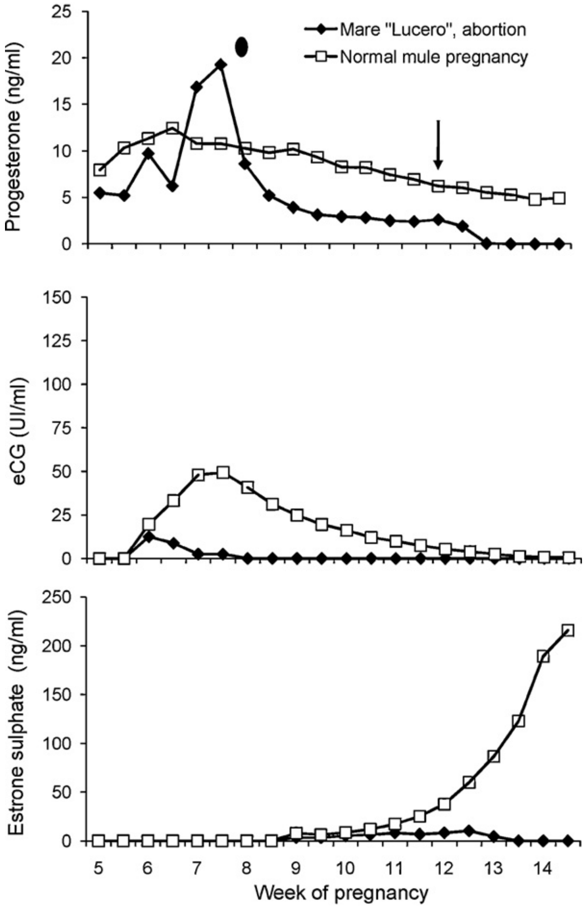

Fig. 4. Progesterone, eCG and estrone sulphate concentrations in a mare that aborted a mule fetus after a period of luteal deficiency secondary to the absence of eCG secretion. This mare also suffered from bacterial placentitis derived from a lesion in the vulva that became infected. The arrow indicates the time of fetal death. The black oval at Week 7.5 indicates the formation of an accessory corpus luteum.

centrations well below those for normal pregnancy with equine fetuses. Estrone sulphate concentrations were lower than normal during the two weeks preceding the abortion. Necropsy of the fetus revealed placental vascular alterations suggestive of hypoxia. No evidence of an inflammatory process was found.

4. Discussion

The similar incidence of embryo mortality (before Day 40 of pregnancy) between mares impregnated with donkey semen (6.2%) and with stallion semen (9.1%) suggests that the endocrine milieu during the first 6 weeks of pregnancy is similarly adequate in both types of pregnancy. This is also suggested by the similar progesterone concentrations between both groups up to Week 6 of pregnancy. Although eCG concentrations between both types of pregnancy became different ($P < 0.05$) on Week 6, this only resulted in lesser ($P < 0.05$) progesterone concentrations in mares with mule pregnancies after Week 8 (Fig. 1).

In contrast, in the present study the incidence of abortion after Day 40 in mares carrying a mule product was 6 times greater than that of mares carrying a horse fetus (30% compared to 5%). The very high incidence of abortions in mares carrying mule fetuses has been observed before (Allen, 1984; Boeta and Zarco, 2005), but the relative frequency of the different endocrine alterations differs between studies.

While no cases of abortions associated with secondary luteal deficiency were found in mule pregnancies in our previous work (Boeta and Zarco, 2005), in the present study luteal impairment secondary to eCG deficiency was the most common finding in mares that aborted mule fetuses. The four cases varied from two mares with the absence of eCG associated with low progesterone concentrations for several weeks before occurrence of abortion (Figs. 3 and 4), to a mare (not shown) with reduced eCG concentrations (less than 30 IU/ml) associated with a very gradual reduction of progesterone concentrations until basal concentrations were reached immediately before abortion (not shown). Chronic progesterone deficiency may have affected placental development and fetal well-being in mares with secondary luteal deficiency, as suggested by

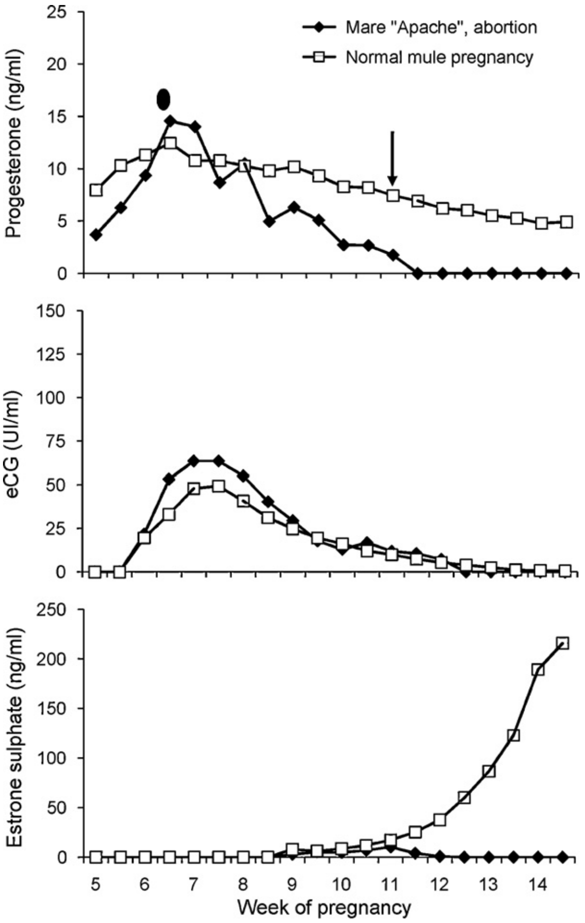

Fig. 5. Progesterone, eCG and estrone sulphate concentrations in a mare carrying a mule embryo and experiencing abortion associated with primary luteal deficiency. The arrow indicates the time of fetal death. The black oval at Week 7 indicates the formation of a supplementary corpus luteum.

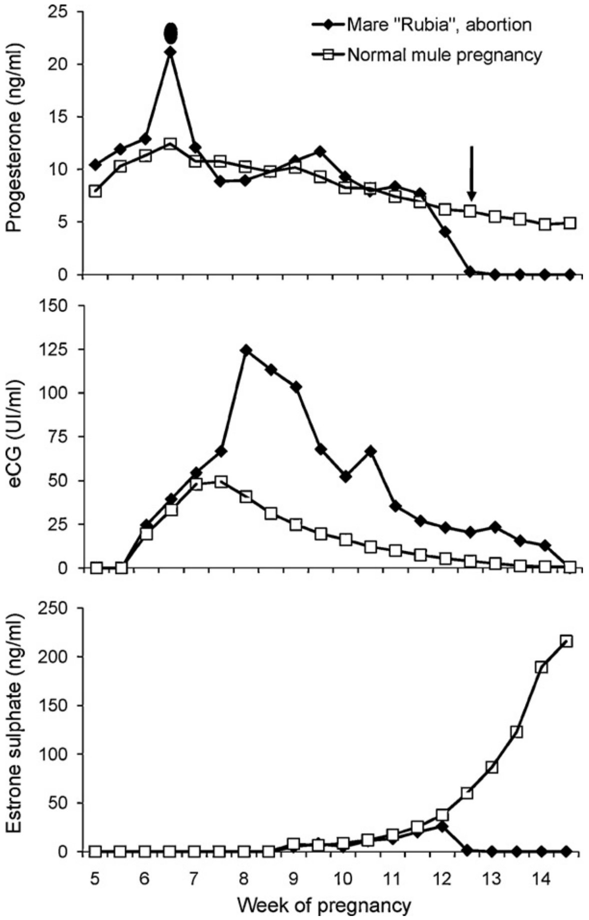

Fig. 6. Progesterone, eCG and estrone sulphate concentrations in a mare carrying a mule embryo and experiencing abortion due to sudden luteal regression (active luteolysis). The arrow indicates the time of fetal death. The black oval at Week 7 indicates the formation of a supplementary corpus luteum.

low estrone sulphate concentrations during the weeks preceding abortion. Terqui and Palmer (1979) demonstrated that normal estrone sulphate production in the pregnant mare requires both the presence of a viable fetus producing normal amounts of dehydroepiandrosterone (DHEA) and a functional placenta capable of normal androgen aromatization. However, in the present study no alterations were found during histopathology of the placenta or fetuses from mares with secondary luteal deficiency that aborted mule fetuses. Interestingly, vascular alterations were found in the placenta of the only mare impregnated with horse semen that aborted, which also appeared to suffer from secondary luteal deficiency.

Mares carrying mule fetuses produce less eCG than mares impregnated by horses (Boeta and Zarco, 2005), and that this is due to premature rejection of the endometrial cups (Allen, 2001a). However, the causes of even lower eCG secretion in some mares that abort mule fetuses are not known, although there appears to be a genetic component since several mares have been identified that fail to produce eCG in successive pregnancies, even when impregnated by horses (Rajkumar et al., 1987; Boeta and Zarco, 2005). Mares maintain their rank in relation to eCG concentrations in successive pregnancies even when mated to different stallions (Martinuk et al., 1990). Genetic susceptibility could explain the absence of abortions associated with secondary luteal deficiency in our previous study (Boeta and Zarco, 2005), because the mares used in each study were purchased from different providers and thus were genetically unrelated.

Primary luteal deficiency in the face of apparently normal eCG secretion (Figs. 2 and 5) occurs in mares that abort mule fetuses (Boeta and Zarco, 2005). The causes of primary luteal deficiency are not known, but could be due to defective formation and/or vascularization of the CL, or to relative insensitivity to LH/eCG. In this regard, eCG concentrations of these mares were not different ($P < 0.05$) than those found during normal mule pregnancies (Fig. 2), they still were about 10 times less than the eCG concentrations found during normal pregnancies with horse fetuses (Fig. 1). Thus, average eCG concentrations during mule pregnancies might be marginally adequate, and in some

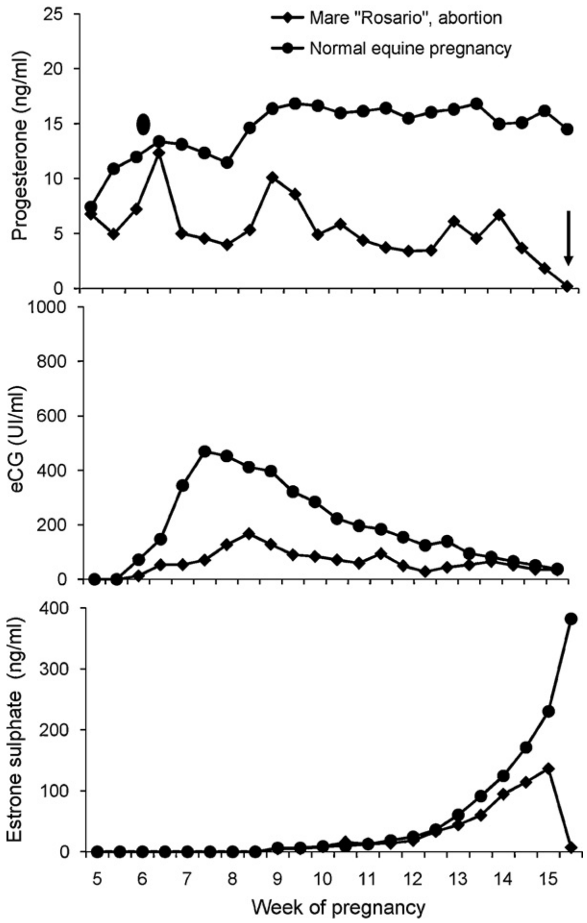

Fig. 7. Progesterone, eCG and estrone sulphate concentrations in a mare that aborted an equine fetus after a period of secondary luteal deficiency. Arrows indicate the time of fetal death. The black oval at Week 6 indicates the formation of an accessory corpus luteum.

individuals these concentrations may not be sufficient to sustain an adequate luteal function.

We have previously reported six cases of abortions associated with an abrupt cessation of luteal function, four of them in mares carrying mule pregnancies (Boeta and Zarco, 2005), and here we report three more cases. The speed and magnitude of the progesterone decrease that immediately preceded these fetal deaths is suggestive of luteal regression due to active PGF2α secretion (Daels et al., 1989, 1991). Furthermore, estrone sulphate concentrations before the three cases reported here were normal and decreased abruptly at the time of fetal death, reinforcing the concept of a sudden catastrophic event.

Exogenous administration of endotoxins induces PGF2α secretion and abortion in pregnant mares (Daels et al., 1991). Presence of eCG protects the CL of pregnancy against the luteolytic effects of PGF2α (Allen, 2001b). Thus, lesser eCG concentrations in mares pregnant with mule fetuses could render them more susceptible to luteal regression resulting from PGF2α secretion induced by traumatic or infectious events, such as an endotoxemia due to colic (Boeta and Zarco, 2005). In this respect, in preliminary studies our group has found that the pregnancy CL of mares impregnated by donkeys are much more susceptible to exogenous PGF2α administration than those of mares impregnated by horses (Velazquez, 2008; Flores, 2009). Furthermore, one of the cases reported in the present study was associated with focal necrotic hepatitis of the fetus, which is compatible with an infectious process capable of inducing PGF2α secretion (Daels et al., 1991). This could result in luteal regression in a mare that was not protected by the high eCG concentrations found in mares with horse fetuses.

It is concluded that the greater incidence of abortion in mares impregnated by donkeys is associated with different kinds of luteal malfunction. Deficiency of eCG secretion may be at the root of many of these cases, either by failing to stimulate enough luteal progesterone secretion and/or by failing to protect the CL of pregnancy from endogenous PGF2α secretion.

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Capacitation status of stored boar spermatozoa is related to litter size of sows

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ABSTRACT

Semen parameters can be considered useful predictors of sperm fertility. The objective of this study was to address the question of whether differences in *in vivo* fertility after the use of different ejaculates could be predicted using sperm kinematics, capacitation status, and sperm penetration ability under commercial pig production conditions. The percentage of capacitated sperm, as assessed by chlortetracycline (CTC) staining, was positively correlated with litter size ($p < 0.01$). Our data suggest that litter size increases in proportion to the number of capacitated spermatozoa. When all semen parameters (kinematics, sperm capacitation status, and sperm penetration ability) and litter size were included in a multiple linear regression analysis, significant associations were found between the percentage of capacitated sperm (B-type), the sperm fertility index as assessed by a sperm penetration assay (SPA), and litter size. This relationship between capacitated sperm and litter size, however, was more predictive for smaller litter groups than larger ones. We found that the percentage of B-type sperm was significantly correlated with historic average litter size. However, there was no significant correlation between the percentage of B-type sperm and historic farrowing rates. To determine the normal range for B-type sperm, the lower limits were established as 30% for small litters (<8 piglets) and 35% for large litters. The overall accuracy of the assay was 92% and 83% for small and large litters, respectively. These results indicate that capacitation status as measured by CTC staining is a useful predictor of sperm fertility, equivalent to SPA. Moreover, original capacitation status exhibited better predictive ability for small litters than for large ones. Therefore, subfertile boars can be identified primarily by capacitation status.

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1. Introduction

The prediction of sperm fertility is of paramount importance for breeding herds when artificial insemination (AI) is used (Gadea, 2005). Initial evaluation of animal sperm fertility generally depends upon semen analyses. While these tests provide valuable data, they yield no information concerning the functional competence of the sperm (Johnson et al., 1990; Petrunkina et al., 2007).

To make up for the weak points of conventional semen analyses, several tests relating sperm function and fertility have been developed for animal species of economic importance, as well as for humans. However, most such tests show relatively little correlation with sperm function and fertility (Gadea and Matas, 2000; Johnson et al., 2000; Rodriguez-Martinez, 2003; Aitken, 2006). There is, thus, an urgent need for new sperm function parameters that correlate better with *in vivo* fertility (Petrunkina et al., 2007). The use of a simple, inexpensive, and accurate assay would be especially valuable under commercial field conditions.

The current emphasis is on tests that use multiple variables to examine how different sperm parameters interact to determine fertility. In this study, we determined which

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measured sperm parameters (sperm kinematics, capacitation status, and sperm penetration ability) are best at discriminating between high- and low-fertility boars.

2. Materials and methods

All procedures were performed, following the approved guidelines for the ethical treatment of animals, according to the regulations of Chung-Ang University (AEC-20080428-2), in compliance with standard international regulations.

2.1. Semen sources

Semen from AI stud boars was collected twice per week, and the entire ejaculate was collected. The low limit values for AI and semen analysis were as follows: volume > 150 ml, total concentration > 200×10^6, motility > 70%, and total abnormalities < 20% of the sample. Commercial AI doses from Duroc boars of proven fertility were diluted to 30×10^6 sperm cells/ml in 100 ml of commercial extender (BTS). Prepared doses of semen were cooled down to 17 °C and transported to the nucleus of the sow pool, where it was stored in a semen storage unit at 17 °C until insemination. Once semen was extended, it was inseminated within 3 days after collection. Extended semen for experiments was used within 2 h of collection.

Historic fertility information was provided by Darby Genetics, Inc. (Ansung, Gyeonggi-Do, Korea) and was analyzed to select 24 Duroc boars (mean age: 38.08 ± 11.74, range = 22–67 months) spanning a wide fertility range (Table 1).

None of the quantitative semen parameters showed significant differences between boars yielding large and small litter sizes. However, historical data indicated that these groups differed significantly in their *in vivo* fertility (Table 1). Although the numbers of inseminations (sows) was small in some boars, the standard errors for farrowing rate and litter size did not deviate much from those found for the group with large numbers of inseminations. Farrowing rate and litter size were used as *in vivo* fertility parameters. The average farrowing rates were calculated for each boar as the percentage of sows inseminated that successfully farrowed. The average litter size for each boar was calculated as the total number of piglets born (live and dead) from each farrowing, averaged across all farrowings (Braundmeier et al., 2004). Each boar was bred with, on average, 22.46 sows and produced 8.96 litters per boar. Sows were inseminated twice per estrus (after the sows showed signs of pro-estrus, the sows were examined for the onset of standing estrus every 6 h by using a back pressure test in the presence of a mature boar), and three billion sperm were deposited in the cervix at insemination. The first insemination was performed within 24 h of estrus detection, and after 24 h of the first insemination, a second insemination completed the whole course of AI. Fertility was measured for every ejaculate as the percentage of sows farrowing after AI. For each sow that farrowed, the number of dead and live piglets was counted, and the sum was defined as the litter size. Duroc sows were bred with semen collected from the 24 boars and inseminated twice per estrus by well-trained technicians.

In this study, a total of 1263 multiparous (two to four pregnancies) Duroc sows were used to evaluate the fertility prediction abilities of different sperm assays. A single ejaculate was considered to be the experimental unit. First, seminal parameters were evaluated and differences between boars were investigated. Second, correlation and logistic regression analyses were performed to detect any significant relationships between semen variables, farrowing rate, and litter size.

Historic fertility data was collected from 2002 to 2006. To determine the normal range of results and calculate the accuracy of chlortetracycline (CTC) staining, semen samples from 24 Duroc boars were used.

2.2. Computer-assisted sperm analysis (CASA)

Sperm motility (%) and sperm motion kinematics were assessed using a computer-aided sperm analyzer (SAIS plus version 10.1, Medical Supply, Seoul, Korea). Briefly, 10 μl of sample (2×10^6 cells/ml) was placed in a Makler counting chamber (Sefi-Medical, Haifa, Israel). The filled chamber was placed on a heated stage at 38.5 °C. Using a 10× objective in phase contrast, the image was relayed, digitized, and analyzed with SAIS software. The movement of at least 250 sperm cells was recorded from random fields (>5) for each sample. The user-defined settings for the program were as follows: frames acquired, 20; frame rate, 30 Hz; minimum contrast, 7; minimum size, 5; low/high size gates, 0.4–1.5; low/high intensity gates, 0.4–1.5; non-motile head size, 16; non-motile brightness, 14. With respect to the motility setting parameters, objects with a curvilinear velocity (VCL) > 10 μm/s were considered motile. Six motility parameters were analyzed.

2.3. Combined Hoechst 33258/chlortetracycline fluorescence assessment of spermatozoa

The dual staining method was based on that described by Perez et al. (1996), with some modifications. Briefly, 135 μl of semen (2×10^6 cells/ml) was added to 15 μl of H33258 solution (150 μM in Dulbecco's phosphate buffered saline, D-PBS) and incubated at 37 °C for 10 min in a light-shielded water bath. Excess dye was removed by layering the mixture over 250 μl of 2% (w/v) polyvinylpyrolidone in D-PBS. After centrifuging at 400 × *g* for 10 min, the supernatant was discarded and the pellet was resuspended in 700 μl D-PBS; 500 μl of this solution were added to 500 μl of a freshly prepared CTC solution (750 mM CTC in 5 ml buffer: 20 mM Tris, pH 7.8 supplementer with 130 mM NaCl and 5 mM cysteine, was prepared daily and kept in the dark at 4 °C). After 20 sec, the reaction was stopped by the addition of 10 μl of 12.5% (v/v) glutaraldehyde solution in 1 M tris buffer and maintained at 4 °C in the dark until evaluation (within 24 h of preparation). Samples were observed under a Nikon microphot-FXA under epifluorescence illumination at X 400 magnification with UV BP 340-380/LP 425 and BP 450-490/LP 515 excitation/suppression filters for H33258 and CTC, respectively. Spermatozoa were classified as dead (D-type; nuclei showed bright blue fluorescence over the sperm head), live/non-capacitated (F-type; bright green fluorescence distributed uniformly

Table 1
In vivo fertility outcomes and semen parameters for each of the 24 boars.

ID	No. of sows	Farrowing rate (%)	Litter size	Mot (%)	B	AR	SFI
A	27	100.00 ± 0.00	5.50 ± 0.39	71.23	24.00	11.68	1.2
B	6	100.00 ± 0.00	6.38 ± 0.24	37.34	22.23	15.03	1.2
C	26	100.00 ± 0.00	6.80 ± 0.60	83.81	27.16	9.95	1.2
D	42	100.00 ± 0.00	7.50 ± 0.94	74.80	14.28	10.54	0.8
E	12	75.44 ± 0.61	7.69 ± 0.69	88.53	23.39	12.96	0.7
F	8	100.00 ± 0.00	7.70 ± 0.12	75.59	20.89	12.84	1.1
G	74	33.78 ± 4.03	7.81 ± 0.64	73.65	20.50	18.36	1.1
H	153	58.17 ± 3.66	8.19 ± 0.42	74.16	28.66	34.48	1.3
I	82	37.80 ± 3.98	8.58 ± 0.58	73.50	31.67	27.07	1.3
J	98	40.82 ± 4.14	8.75 ± 0.61	74.16	29.52	19.96	1.4
K	16	100.00 ± 0.00	8.98 ± 0.53	73.73	30.63	19.83	1.2
L	33	72.89 ± 1.11	9.00 ± 0.22	83.15	45.27	12.55	2.0
M	42	100.00 ± 0.00	9.20 ± 0.42	91.51	40.09	4.68	1.7
N	84	72.62 ± 1.77	9.23 ± 0.45	73.20	31.69	26.18	2.1
O	115	44.35 ± 4.22	9.41 ± 0.47	64.42	30.74	16.40	1.7
P	33	82.00 ± 0.97	9.50 ± 0.47	86.07	40.91	14.46	1.2
Q	18	100.00 ± 0.00	9.70 ± 0.62	72.17	34.36	18.69	1.2
R	42	76.19 ± 1.09	10.13 ± 0.59	45.85	41.19	21.63	3.3
S	116	43.10 ± 4.33	10.18 ± 0.46	87.36	38.32	22.63	3.0
T	10	100.00 ± 0.00	10.25 ± 0.74	76.42	45.26	9.69	3.9
U	121	100.00 ± 0.00	10.88 ± 0.25	47.80	41.44	10.05	3.3
V	55	100.00 ± 0.00	10.98 ± 0.37	73.61	37.79	17.49	3.2
W	44	100.00 ± 0.00	11.00 ± 0.47	80.09	37.11	16.45	1.7
X	6	100.00 ± 0.00	11.00 ± 0.61	80.30	34.36	14.84	4.0
Mean	15.62	80.72 ± 24.58	8.93 ± 1.20	73.43	32.10	16.60	1.9

Farrowing rate and litter size data are mean ± standard error (S.E.) based on each ejaculate. For semen analysis, all tests were performed using extended semen. Mot = motility (%); B = capacitated sperm; AR = acrosome-reacted sperm; SFI = sperm fertility index.

over entire sperm head with or without a stronger fluorescent line at the equatorial segment), live/capacitated (B-type; green fluorescence over acrosomal region and dark postacrosome), or live/acrosome-reacted (AR-type; sperm showing mottled green fluorescence over the head, green fluorescence only in post-acrosomal region, or no fluorescence over the head) (Maxwell and Johnson, 1999) (Fig. 1). All sperm had bright green fluorescent midpieces. At least 400 spermatozoa were evaluated per sample.

2.4. Sperm penetration assay using zona-free hamster oocytes (SPA)

Hamster oocytes were collected from 12-week-old female after the administration of 30 IU of pregnant mare serum gonadotropin followed 48 h later by injection of 30 IU human chorionic gonadotropin (hCG). Hamsters were killed within 15 h after hCG injection. The cumulus oocyte complexes were removed from the dissected oviducts and treated with 0.1% hyaluronidase and 0.1% trypsin to remove the cumulus cells and zona pellucida, respectively. The oocytes were then washed three times with PBS after the enzyme treatment. Each group of 10 hamster oocytes was co-incubated with 2×10^6 spermatozoa/ml for 3 h in a 4-well dish containing 500 μl of each treatment medium at 39 °C under 5% CO_2 in air. At the end of the co-incubation period, oocytes were mounted on slides, fixed, and then stained with 1% lacmoid.

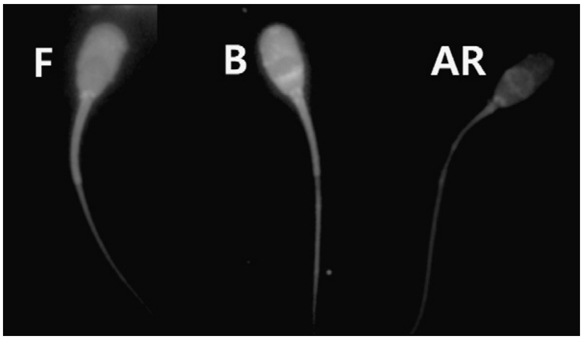

Fig. 1. The three types obtained of chlortetracycline staining observed on live pig spermatozoa. F = live/non-capacitated spermatozoa, B = live/capacitated spermatozoa, and AR = acrosome-reacted spermatozoa.

The data obtained from the penetration assays were expressed as an index that we have termed the sperm fertility index (SFI; no. of enlarged sperm heads + no. of decondensed sperm heads + 2 × [no. of pronuclei] per ovum), which weights the different scores according to their activation in the oocyte. We gave the pronuclei twice the weighting of enlarged and decondensed sperm heads.

2.5. Quality assessment of screening tests

Four key parameters are used to assess screening tests: sensitivity, specificity, positive predictive value, and negative predictive value (Evans et al., 2002). Sensitivity determines the percentage of all the boars that will correctly be identified by the test as producing either small or large litters. Specificity determines what percentage of truly negative boars will test negative. Among all the boars with a positive test result, it is important to know what percentage actually produces small or large litter sizes: this is the positive predictive value. The negative predictive value

Table 2
Correlations between sperm kinematics, capacitation status, sperm penetration ability, and boar *in vivo* fertility.

	Farrowing rate	Litter size	SFI	F	B	AR	Motility	VCL	VSL	VAP	LIN	ALH	HYP
Farrowing rate		0.073	0.173	0.447*	0.121	−0.612**	0.120	−0.442*	−0.070	−0.027	−0.459*	0.154	−0.477*
Litter size			0.726**	−0.445*	0.834**	−0.163	0.010	0.043	−0.099	−0.052	−0.127	0.056	0.003
SFI				−0.182	0.776**	−0.092	−0.099	−0.034	−0.109	−0.058	−0.127	0.015	0.091
F					−0.584**	−0.689**	0.086	−0.315	0.166	0.000	0.694**	−0.128	−0.352
B						−0.030	0.115	0.011	0.008	0.056	−0.026	0.093	0.042
AR							−0.330	0.415*	−0.112	0.000	−0.769*	0.264	0.516*
Motility								0.308	0.576*	0.515*	0.330	0.306	0.011
VCL									0.774**	0.838**	−0.425*	0.920**	0.836**
VSL										0.963**	0.221	0.830**	0.489*
VAP											0.079	−0.893**	0.612**
LIN												0.228	−0.644*
ALH													0.819**

SFI = sperm fertility index; F = non-capacitated sperm; B = capacitated sperm; AR = acrosome-reacted sperm; Mot = motility (%); VCL = curvilinear velocity (μm/s); VSL = straight-line velocity (μm/s); VAP = average path velocity; LIN = linearity; ALH = mean amplitude of head lateral displacement (μm); ^{11}HYP = hyperactivated sperm (%).

* $p < 0.05$.

** $p < 0.01$.

is the percentage of boars with a negative test result that actually have a small or large litter size.

2.6. Statistical analysis

Statistical analyses were performed using a statistical software program (SPSS Version 12.0, Chicago, USA). Spearman correlation coefficients were calculated to determine the association between sperm kinematics, capacitation status, SPA, and boar *in vivo* fertility. Confidence intervals (95% CI) of the correlation coefficients were also provided. Multiple linear regression analysis was used to assess the relationship between semen parameters (capacitation status, sperm kinematics, and SPA) and litter size or farrowing rate. Receiver-operating curves (ROCs) were used to determine the overall discriminant power of each semen parameter in the multiple linear regression model. ROCs were also used to calculate the cut-off value for each semen parameter in relation to the fertility trial. The cut-off value was determined in relation to the point that maximized specificity and sensitivity.

3. Results

Boars used in the study had historic farrowing rates ranging from 34% to 100% and an average litter size ranging from 5.5 to 11.0 pigs/litter. Table 2 shows the simple relationship between measured sperm parameters and the *in vivo* fertility of the boars. A comparison between historic litter size and farrowing rate for each boar revealed a non-significant correlation. Litter size was negatively correlated with the percentage of F-type sperm. The litter size was also positively correlated with the percentage of B-type sperm. The farrowing rate was negatively correlated with AR-type sperm. The percentage of F-type sperm was negatively correlated with the percentage of AR-type sperm. However, motility (%) was not correlated with farrowing rate, litter size, SFI and capacitation status. Of all the variables included in the study, litter size was significantly correlated with F-type and B-type sperm as well as SFI. The farrowing rate was significantly correlated with the percentage of F-type and AR-type sperm, VCL, LIN, and HYP.

A model was designed using sperm kinematics, capacitation status, and sperm penetration ability as independent variables to assess their relationship to litter size (Table 3). When the results for all semen parameters (sperm kinematics, capacitation status, and sperm penetration ability) and litter size were examined using multiple regression analysis, only the percentage of B-type sperm and SFI were significantly associated with litter size ($p < 0.05$). B-type percentage had a greater effect than SFI on litter size. The model showed that these parameters were related to litter size ($R^2 = 0.654$).

Another model was designed using sperm kinematics, capacitation status, and sperm penetration ability as independent variables to assess their relationship to farrowing rate (Table 4). Although these parameters were correlated to farrowing rate in the simple analysis, no relationship to farrowing rate was found in the multiple linear regression analysis ($R^2 = 0.460$).

To determine the normal range for percentage of B-type sperm under CTC staining, a percentage of 30% B-type sperm was established as the lower limit (Fig. 2). Table 5 shows that a positive score (B-type sperm ≤ 30%) with CTC staining is highly predictive of a small litter size (<8). Of boars with a B-type percentage ≤ 30%, 100% produced lit-

Table 3
Multiple linear regression analysis to assess the relationship of sperm kinematics, capacitation status, and sperm penetration ability with litter size.[a].

Model	Estimate (B)	S.E	Beta	*t*-Value	*p*-Value
B-type	0.086	0.031	0.480	2.757	0.012
SFI	0.603	0.261	0.402	2.306	0.031

[a] Estimate represents the regression of litter size on percentage of B-type sperm and SFI. The R^2 for this model was 0.654.

Table 4
Multiple linear regression analysis to assess the relationship of sperm kinematics, capacitation status, and sperm penetration ability with farrowing rate.[a].

Model	Estimate (B)	S.E	Beta	*t*-Value	*p*-Value
F-type	−0.245	0.545	−0.126	−0.450	0.658
AR-type	−0.646	0.809	−0.277	−0.798	0.435
VCL	−0.059	0.297	−0.064	−0.198	0.845
LIN	1.044	0.912	0.403	1.145	0.267
HYP	−1.669	3.712	−0.151	−0.450	0.658

[a] Estimate represents the regression of farrowing rate on acrosomal status and sperm kinematic parameters. The R^2 for this model was 0.460.

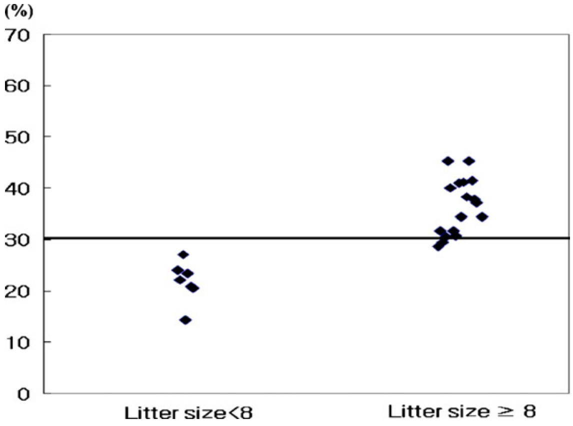

Fig. 2. Determination of the lower limit of normal litter size according to the percentage of B-type sperm under CTC staining. The lower limit of the normal litter size was defined as ≥8.

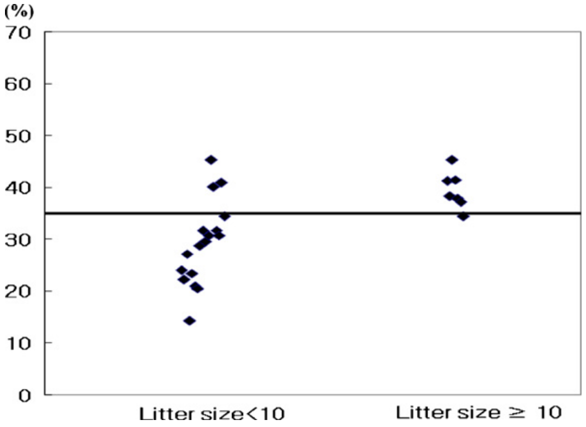

Fig. 3. Determination of the lower limit of large litter size according to the percentage of B-type sperm under CTC staining. The lower limit of the larger litter size was defined as ≥10.

Table 5
Correlation between the percentage of B-type sperm and litter size. Boars with a proportion of B-type sperm ≤ 35% have an increased probability of producing small litters.

	Litter size < 8	Litter size ≥ 8
B-type ≤ 30% (*n* = 9)	7	2
B-type > 30% (*n* = 15)	0	15
Sensitivity		100%
Specificity		88%
Positive predictive value		78%
Negative predictive value		100%
Overall accuracy		92%

ters of fewer than eight piglets. In contrast, of the boars with a B-type percentage > 30%, 88% had litters of eight or more piglets. The overall accuracy for the prediction of large litter size was 92%.

Table 6
Correlation between the percentage of B-type sperm and litter size. Boars with a B-type percentage > 35% have an increased probability of producing large litters.

	Litter size ≥ 10	Litter size < 10
B-type > 35% (*n* = 9)	6	3
B-type ≤ 35% (*n* = 15)	1	14
Sensitivity		86%
Specificity		82%
Positive predictive value		67%
Negative predictive value		93%
Overall accuracy		83%

To determine the cut-off for larger litter size using CTC staining, a percentage of 30% B-type sperm was established as the lower limit (Fig. 3). Table 6 shows that a positive CTC staining score (B-type > 35%) was highly predictive of larger litter size (≥10). With a B-type percentage > 35%, 86% of the boars produced litters containing 10 or more piglets. In contrast, of the boars with a B-type percentage ≤ 35%, 82% had litters of fewer than 10 piglets. The sensitivity defines what percentage of all the boars with large litter size will be correctly identified by the test. Conversely, the specificity defines what percentage of the boars will correctly test negative. Of all the boars with a positive test result (B-type percentage > 35%), it is important to know what percentage actually has large litters (≥10) (positive predictive value). The negative predictive value is just the opposite: the percentage of all boars with a negative test result (B-type percentage ≤ 35%) that indeed have small litters (<10). It is important to note that of the boars with litter sizes of 10 or more, 93% had a B-type percentage > 35%. The overall accuracy of the prediction of large litter size was 83%.

4. Discussion

In this study, to determine the normal range for CTC results, the lower limits of B-type percentage were established as 30% for a small litter size (<8 piglets) and 35% for a large litter size. The overall accuracy of the assay for the prediction of small and large litter sizes was 92% and 83%, respectively. These results indicate that capacitation status as measured by CTC staining is a useful predictor of fertility that is equivalent to our optimized SPA. Moreover, because CTC staining exhibited better predictive accuracy for small litter sizes than for larger ones, subfertile boars could be identified based on their capacitation status.

Because fertilization is a complex probability event comprising several steps, there is no reliable method that can evaluate the complicated interactions that occur from the time of transport of spermatozoa to fertilization (Larsson and Rodriguez-Martinez, 2000). A successful pregnancy is the only irrefutable proof that a sperm is capable of fertilization, but few single function tests truly reflect *in vivo* fertility (Chang et al., 1990; Roldan, 2007). Therefore, Amann and Hammerstedt (1993) and Collins et al. (2007) suggested that a two- or three-tiered approach should be used, and that each test should emphasize the characteristics of individual sperm. In other words, the more sperm parameters that can be tested, the more accurate the test will be.

Numerous methods have been developed over the years for laboratory evaluation of semen quality and fertility. Some of these methods measure general characteristics of the sperm (viability, motility types, morphology, sperm metabolism, membrane, and acrosomal integrity), and among these, semen fertility appears to be more closely related to membrane integrity than to other general characteristics (Zavos et al., 1995; Larsson and Rodriguez-Martinez, 2000). Motility is perhaps the most easily observed function of sperm, yet is arguably the least well-understood (Darszon et al., 2007). An evaluation of motility is the most widely used sperm assessment method, as it is a good indicator of the intactness of the membranes and of functionality. However, the relation between motility and fertilizing ability initially appeared to be contradictory (Galli and Bosisio, 1988; Selles et al., 2003). A CASA was used to resolve this paradox, and a significant correlation with fertility was found (Hirai et al., 2001). CASA has been used to evaluate sperm motility parameters in several species including boars (Holt et al., 1997). Vizcarra and Ford (2006) reported that sperm motility assays can be used for mammalian sperm; a normal distribution in CASA results was apparent and needed to evaluate the capacity to predict fertilizing ability in boars.

Among all the sperm tests that have been developed, IVF tests are the most suitable for assessing overall sperm function during fertilization (Parinaud et al., 1996). Binding to and penetration of the zona pellucida are essential tasks that spermatozoa must complete for fertilization to occur (Harrison, 1997). Furthermore, the interaction of sperm with the oocyte plasma membrane appears to explain much of the variability in sperm fertilizing potential among fertile boars (Berger et al., 1996). Hence, assays that can evaluate gamete interactions may lead to a better way to predict male fertility than routine laboratory evaluations of semen (Bavister, 1990; Larsson and Rodriguez-Martinez, 2000). However, the donation of maturated cumulus cells and oocytes necessary for IVF procedures is not widespread, making these methods costly and time-consuming to implement. IVF tests (Parinaud et al., 1996), homologous *in vitro* penetration (hIVP) tests (Gadea and Matas, 2000), and SPA are more accurate tests for predicting sperm fertilizing ability (Kruger et al., 1988). In our previous study, SPA results showed a significant correlation with historic average litter size but had no significant correlation with farrowing rates. Furthermore, SFI predicted sperm fertility; the overall accuracy was 92% and 96% for small and large litter sizes, respectively (Oh et al., 2009). However, these methods have some limitations, such as requiring time-consuming and expensive experiments. These are problems that need to be addressed, and various methods are being developed for easier and more accurate assessments.

The capacitation status of sperm can be assessed by monitoring calcium-mediated changes using the fluorescent antibiotic CTC. Neutral and uncomplexed CTC traverses the cell membrane of spermatozoa and enters intracellular compartments containing free calcium (Tsien, 1989). The CTC–calcium complex binds preferentially to hydrophobic regions, such as the cell membrane, resulting in different and characteristic staining of non-capacitated, capacitated, and acrosome-reacted spermatozoa (Gillan et al., 2005). These methods have been successfully used in numerous species, including mice (Saling and Storey, 1979; Ward and Storey, 1984), humans (Lee et al., 1986), and rams (Gillan et al., 1997) and were validated for boar spermatozoa (Wang et al., 1991; Mattioli et al., 1996).

Cryopreserved boar spermatozoa demonstrate a significant reduction in conception rates compared with liquid semen when used for artificial insemination. It is generally accepted that preliminary stages of cryopreservation of spermatozoa involving cooling to 5 °C have been shown to induce capacitation-like changes in boar spermatozoa (Bravo et al., 2005). Therefore, a larger proportion of these capacitated sperm present in the semen at the time of insemination is considered to have an adverse effect on fertility in porcine. This is based on the assumption that prematurely capacitated sperm present in the insemination may survive in the female reproductive tract only for a short period, reducing fertility. In our previous study (Park et al., 2008), liquid boar semen samples extended in BTS and stored at 17 °C up to 5 days. A significant increase in the proportion of AR-type spermatozoa and a corresponding significant decrease in the proportion of B-type spermatozoa were found in 4-day stored samples, whereas there was no change in the proportion of F-type spermatozoa with time of storage. Shifting from a larger proportion of B-type spermatozoa to AR-type suggest that most of capacitated spermatozoa readily undergo physiological transformation to AR. Therefore it is conceivable that most of B-type spermatozoa in our condition supposedly differentiate from premature capacitation after cooling to 5 °C, though it was not confirmed by changes in tyrosine phosphorylation (Green and Watson, 2001; Bravo et al., 2005).

We undertook the present study to determine which measured sperm parameters are best at discriminating between high- and low-fertility boars. To do this, simple correlations between measured sperm parameters and the historic *in vivo* fertility of boars were tested. The results of this study demonstrate that the percentage of capacitated sperm, as assessed by CTC staining, is significantly correlated with litter size in pigs ($p < 0.01$). Our data suggest that litter size directly increases with the proportion of capacitated spermatozoa. When all semen parameters (sperm capacitation status, kinematics, and sperm penetration ability) and litter size were included in a multiple linear regression analysis, significant associations were found between the percentage of capacitated sperm as assessed by CTC staining, SFI as assessed by SPA, and litter size. This relationship between capacitated sperm and litter size, however, was more predictive of smaller litter groups than larger ones. Thus, individual differences between boars in their initial capacitation status may play a decisive role in the identification of subfertility in pigs.

We found that the percentage of B-type sperm had a significant correlation with historic average litter size. However, the percentage of B-type sperm had no significant correlation with historic farrowing rates. In contrast, the farrowing rate was significantly correlated with the percentage of F-type sperm, VCL, LIN, and HYP ($p < 0.05$). The percentage of AR-type sperm was negatively correlated with farrowing rate. A sperm cell must retain a normal acrosome so that the acrosome reaction can occur at the proper time to facilitate fertilization. The vesiculation of the acrosomal and plasma membranes that occurs during cell death is termed a false acrosome reaction. A true acrosome reaction, which precedes fertilization, occurs only in live intact sperm. Thus, it is important not only to analyze semen for sperm viability, but also to determine the normalcy of the acrosome simultaneously (Thomas et al., 1997).

None of the sperm kinematic measurements were significantly correlated with litter size. However, farrowing rate was significantly related to VCL, LIN, and HYP. With regard to the relationship between sperm kinematics and *in vivo* fertility, Sutkeviciene et al. (2005) showed that only three sperm quality parameters correlated with the non-return rate: the computer-assessed total sperm motility in semen after 7 days of storage, the subjective motility in semen after 7 days of storage, and the computer-assessed total motility of methanol-stressed spermatozoa after 1 day of storage. However, the correlation coefficients between these sperm quality parameters and non-return rate and litter size were not very high.

Xu et al. (1998) suggested that litter size may be a more accurate measurement of sperm fertility than farrowing rate. Braundmeier et al. (2004) also suggested a possible explanation for this phenomenon: measurement of farrowing rate only gives two possible outcomes, pregnant or not, whereas litter size measurements give a wide range of outcomes. This range may allow the use of litter size to more accurately gauge the fertility status of boars.

5. Conclusions

To compensate for the weak points of conventional semen analyses, several tests that evaluate sperm function and fertility have been developed for economically important animal species and humans. Unfortunately, these methods require bioassays that are complicated, time-consuming, and expensive. The identification of other methods to assess sperm function, which provide the same information but are easier to perform, would make fertilization assays both unnecessary and impractical. The identification of such tests would require advances in both laboratory tests for sperm function and the definition of the best conditions for fertility evaluation. The use of a simple, non-expensive, and accurate assay would be especially valuable under commercial field conditions. In light of the above, the capacitation status of sperm as measured by CTC staining can be considered a useful predictor of fertility that is equivalent to our optimized SPA. Moreover, because CTC staining exhibited better predictive accuracy for smaller litter sizes than for larger ones, subfertile boars could be identified based primarily on their capacitation status.

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Reproductive performance of gilts according to growth rate and backfat thickness at mating

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ABSTRACT

The study evaluated the influence of growth rate (GR) and backfat thickness (BF), at first mating of gilts, on the reproductive performance until the first farrowing and on the variation in birth weight of piglets. Gilts were categorized into three groups according to GR from birth until the first mating: GRI (600–700 g/d; $n = 345$), GRII (701–770 g/d; $n = 710$) and GRIII (771–870 g/d; $n = 366$). Analyses were also performed considering three groups formed according to BF (mm) at mating: BFI (10–15 mm; $n = 405$); BFII (16–17 mm; $n = 649$) and BFIII (18–23 mm; $n = 367$). There were no differences in farrowing rate and return to estrus rate among BF or GR groups ($P > 0.05$). GRII and GRIII females had larger litter size compared to GRI gilts ($P < 0.05$), respectively, 0.5 and 0.9 more piglets, but a greater percentage of intra-partum stillborns ($P < 0.05$) was observed in GRIII than in GRI and GRII females. Moreover GRIII females had more piglets ($P < 0.05$) weighing less than 1,200 g, litters with a greater coefficient of variation for birth weight and a greater percentage of litters with a coefficient of variation above 20% ($P < 0.05$) than GRI females. More total born and born alive piglets were observed in BFII compared with BFI females ($P < 0.05$). There were no differences among BF groups in number of stillborn neither in variables concerning the birth weight of piglets ($P > 0.05$). These results show that there is no advantage, in terms of farrowing rate and number of born alive, in performing the first mating of gilts with GR > 770 g/d and BF > 17 mm.

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1. Introduction

To achieve breeding targets and to make gilts as highly productive as possible, it is important to recognize the key physiological characteristics of contemporary dam-line females, and particularly the exceptional lean growth potential of the modern genotypes (Foxcroft et al., 2005; Bortolozzo et al., 2009). Positive relationships of growth rate (GR) or backfat thickness (BF) with the subsequent gilt reproductive performance occurred in several studies (Tummaruk et al., 2001, 2007; Kummer et al., 2006), although there are inconsistencies (Rozeboom et al., 1996).

With unrestricted feeding and recommended space allocations (~1.5 m^2 per gilt) during development, growth rate does not seem to limit the age at the onset of first estrus (Foxcroft et al., 2005). Greater growth rate gilts can have the pubertal estrus successfully induced before 150–170 days of age (Amaral Filha et al., 2009) and gilts can be bred between 185 and 210 days of age (Kummer et al., 2006) without impairing the reproductive performance. In gilts bred at third estrus, the variation in growth rate resulted in an enormous variation in weights at breeding, ranging from 100 to 190 kg (Foxcroft, 2005), showing that gilts with greater growth rates may become overweight by the time they are bred, if a predetermined moment (third estrus

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in this case) is taken into account for the first breeding. Thus, traditional management practices need to be reevaluated if we are to capture the full economic potential of the modern breeding gilt (Foxcroft et al., 2005), even if it is difficult to discern the effects of age, weight, backfat and estrus number at first mating on subsequent reproductive performance and longevity (Bortolozzo et al., 2009).

The objective of this study was to investigate, through a retrospective analysis, the influence of GR and BF at the first mating of contemporary dam-line gilts on the subsequent reproductive performance and on birth weight variation of the first litter. The study was conducted during the stocking of a farm, which represented a good opportunity to verify this effect in a large number of females.

2. Materials and methods

2.1. Animals, facilities and general management practices

The study was performed from June 2006 to April 2007 in a farm with a capacity to accommodate 2,400 females. The farm is located in the midwest of Brazil (parallel 14°), a region with a tropical climate. The natural photoperiod of this region is around 12 h of darkness and 12 h of lighting, and no extra light was provided.

The study comprised 1,421 PIC Camborough 22® line gilts. They were fed *ad libitum* prior to being shipped to the farm (on average with 146 ± 0.2 days of age). Water was provided *ad libitum* to females throughout the study.

After farm arrival, gilts were housed in pens as groups of 12 gilts per pen. Pens had compacted concrete floor and provided a space allowance of 1.5 m^2 per gilt. Gilts remained in individual crates (0.6 m × 2.5 m) with partially slatted concrete floor and an automatic feeding system, from approximately 20 days before AI until being transferred to farrowing house, which took place 3–5 days before predicted farrowing. The farrowing rooms consisted of four rows with seven farrowing crates in each. Crates were equipped with water nipples and semi-automatic feeders and had a full plastic slatted floor. All these facilities had fans and sprinklers, which were turned on when the indoor temperature exceeded 25 °C.

During the puberty stimulation period, which started when gilts were on average 150 ± 0.3 days old, they received a total amount of 3.5 kg of feed per day which was offered divided over three meals, based on a standard corn soybean diet (3,200 kcal ME/kg, 16.9% CP and 0.9% lysine). For puberty stimulation, gilts were exposed to a sexually mature boar (>12 months of age), which was introduced into each pen twice a day for 20 min. A rotation of boars was performed by replacing those used in the morning by other ones in the afternoon. Gilts had full physical contact with boars and were considered in estrus if they showed a standing reflex in response to the backpressure test in the presence of the boar.

About 20 days before AI, gilts were submitted to flush feeding. A total of 3.5 kg of feed based on a corn soybean diet (3,330 kcal ME/kg of ME, 19.4% CP and 1.1% lysine) was offered per day, divided over three meals. Gilts were inseminated on average with 217.9 ± 0.26 days of age and 3.04 ± 0.02 recorded estrus periods after the onset of boar exposure. The first AI was performed around 12 h after the onset of estrus and then every a.m. and p.m. until they were no longer standing. Gilts were inseminated on average 3.2 ± 0.01 times with pooled semen doses containing 3 billion sperm cells diluted in 90 mL of BTS (Beltsville Thawing Solution—MINITUB®) extender. The semen doses were stored at 15–18 °C and none semen dose was used for AI after 72 h of storage.

During pregnancy, gilts were automatically fed twice a day, with 1.8, 2.0, 2.2 and 2.4 kg of feed per day at 0–5, 6–30, 31–60 and 61–90 days of gestation (day 0 = day of first AI), respectively, with a corn soybean diet (2,900 kcal ME/kg, 14% CP and 0.77% lysine). From 91 to 110 days of gestation gilts were fed twice a day with 2.8–3.0 kg of a pre-lactation feed (3,153 kcal ME/kg, 17.0% CP and 0.9% lysine), with a gradual reduction in the quantity provided, from 3 kg (5 days before the expected farrowing) to 1 kg (1 day before the expected farrowing).

2.2. Data collection

Data collection and measurements were performed by the research team with the help of farm personnel. Body weight and BF were measured at AI (2–3 days before) and at farrowing. The GR from the birth until the first insemination was calculated by dividing the weight by age, without adjusting for birth weight. At the level of the last rib, at approximately 6 cm from the midline, BF was measured by A-mode ultrasonography (Renco lean meter—Renco Corporation, Minneapolis, MN), on both sides of the female (Tummaruk et al., 2009). The mean obtained from these two measurements was the value used in the analysis.

Pregnancy testing was done by return to estrus control starting day 15 and performed until day 100 of gestation, and the percentage of animals returning to estrus was then calculated over the total of inseminated gilts. Adjusted farrowing rate (AFR) was calculated after excluding dead sows and sows removed by non-reproductive reasons.

Parturitions were supervised and sows were individually weighed and BF was measured within 24 h after parturition. Piglets born alive and stillborn were counted and individually weighed within 12 h after birth, and the mummified fetuses were counted and included in the total number of born piglets. After necropsy, stillborn piglets were classified as prepartum and intra-partum. Prepartum stillborn were those that died a few days before parturition (Muirhead and Alexander, 2000), when organs were autolytic with evidence of embedded hemoglobin pigment (Sims and Glastonbury, 1996). Both prepartum and intra-partum stillborns showed signals of no breathing when evaluated for lung inflation (Muirhead and Alexander, 2000).

2.3. Study design and statistical analyses

The gilts were retrospectively classified into groups of GR between birth and first mating and BF at mating, respectively: GRI (600–700 g/d; n = 345), GRII (701–770 g/d; n = 710) and GRIII (771–870 g/d; n = 366); BFI (10–15 mm; n = 405), BFII (16–17 mm; n = 649) and BFIII (18–23 mm;

Table 1
Descriptive statistics of gilts according to their growth rate (GR) between birth and first mating, and backfat thickness (BF) at first mating (means ± SEM).

	GR, g/d	Weight, kg	BF, mm	Age, d
Growth rate, g				
GRI (600–700) ($n = 345$)	673 ± 1.14 (602–700)	147.4 ± 0.46 (130–167)	15.8 ± 0.09 (11–22)	219.0 ± 0.53 (196–240)
GRII (701–770) ($n = 710$)	735 ± 0.79 (701–770)	160.4 ± 0.32 (134–184)	16.4 ± 0.07 (11–23)	218.4 ± 0.37 (188–240)
GRIII (771–870) ($n = 366$)	802 ± 1.11 (771–869)	173.2 ± 0.45 (154–199)	17.0 ± 0.09 (10–23)	215.9 ± 0.52 (194–240)
Backfat thickness, mm				
BFI (10–15) ($n = 405$)	722 ± 2.44 (615–864)	157.0 ± 0.61 (130–193)	14.2 ± 0.04 (10–15)	217.6 ± 0.49 (195–239)
BFII (16–17) ($n = 649$)	737 ± 1.93 (602–869)	160.7 ± 0.48 (131–199)	16.5 ± 0.03 (16–17)	218.2 ± 0.39 (194–240)
BFIII (18–23) ($n = 367$)	754 ± 2.56 (619–868)	164.2 ± 0.64 (132–197)	18.6 ± 0.04 (18–23)	217.7 ± 0.52 (188–240)

Range values are shown within parenthesis.

$n = 367$). The categories of GR and BF were established according to limits close to the values observed in the distribution of first, second to third, and fourth quartiles for Groups I, II and III, respectively.

All the statistical analyses were performed with Statistical Analysis System software, version 9.1.3 (SAS, 2005) and the significance level used in the comparisons among groups was 5%. Descriptive statistics concerning GR from birth until AI, age at AI, weight at AI (Table 1), and estrus number at AI were obtained by using the MEANS procedure. Correlation coefficients between body weight and BF, at mating and at farrowing, were obtained with the CORR procedure. Net weight gain during pregnancy, weight and BF at farrowing, number of total born and number of born alive piglets were analyzed by the GLM procedure and least squared means (Lsmeans) were compared by the Tukey–Kramer test. The effect of BF groups on the number of total born piglets was adjusted by the inclusion of GR as a covariable. However, BF was not significant as a covariable in the model used to investigate the effect of GR groups on total born piglets. Percentages of mummified fetuses and stillborn (total, prepartum and intra-partum) piglets were analyzed by the non-parametric NPAR1WAY procedure and compared by the Kruskal–Wallis test. Return to estrus rate, AFR and percentage of females with stillborns were compared by the Chi-square test.

Data of 1,094 litters were used for the analysis of the coefficient of variation (CV) for birth weight within litters. The birth weight (of born alive and stillborn piglets), the CV of birth weight and the number of piglets weighing less than 1,200 g were analyzed with the GLM procedure. Percentages of litters with a CV for birth weight higher than 20% were compared by the Chi-square test.

3. Results

Results of the reproductive performance according to GR groups are shown in Table 2. There were no differences ($P > 0.05$) in return to estrus rate and AFR among GR groups. Sows of GRII and GRIII groups had greater number of total born ($P < 0.05$) compared to GRI sows but there were no differences ($P > 0.05$) among groups in the number of born alive piglets. Females of GRIII group had a greater percentage of total and intra-partum stillborns ($P < 0.05$) compared to GRI and GRII groups.

Results of the reproductive performance according to BF groups are shown in Table 3. There were no differences ($P > 0.05$) in return to estrus rate and AFR among BF groups. Females of BFII group had a greater number ($P < 0.05$) of total born and born alive piglets than BFI females. The numbers of total born and born alive piglets of BFIII females did not differ ($P > 0.05$) from those observed in BFII and BFI females. Percentages of total and intra-partum stillborns were not different ($P > 0.05$) among BF groups.

Overall, the percentages of prepartum stillborns and mummified fetuses were 1.0% and 3.2%, respectively, and they were not different ($P > 0.05$) among GR or BF groups.

More piglets ($P < 0.05$) weighing less than 1,200 g were produced by GRIII than GI sows (Table 4). A lesser CV of birth weight was observed in GRI litters compared to GRII and GRIII groups. GRIII females had a greater percentage of litters with a CV of birth weight above 20%

Table 2
Fertility results of gilts according to their growth rate (GR) between birth and first mating (LSmeans ± SEM).

	Growth rate groups, g/d		
	GRI (600–700)	GRII (701–770)	GRIII (771–870)
Return to estrus rate, % (n/n)	6.4 (22/345)	6.2 (44/710)	6.0 (22/366)
Farrowing rate, % (n/n)	91.3 (315/345)	91.7 (651/710)	92.6 (339/366)
Adjusted farrowing rate[c], % (n/n)	92.6 (315/340)	92.7 (651/702)	93.6 (339/362)
Total born piglets, n	12.0 ± 0.16 a	12.5 ± 0.11 b	12.9 ± 0.16 b
Born alive piglets, n	10.9 ± 0.17	11.3 ± 0.12	11.3 ± 0.16
Total stillborn[d], % (Q1–Median–Q3)	5.5 ± 0.61 (0–0–8.3) a	6.1 ± 0.44 (0–0–8.3) a	8.7 ± 0.83 (0–5.6–12.5) b
Intra-partum stillborn[d], % (Q1–Median–Q3)	4.7 ± 0.59 (0–0–7.7) a	5.1 ± 0.41 (0–0–7.7) a	7.2 ± 0.74 (0–0–9.1) b

a,b: Different letters indicate statistical differences in the same row ($P < 0.05$).

[c] Calculated by excluding dead females and females removed for non-reproductive reasons. Overall abortion and failure to farrow rates were, respectively, 0.63% (9/1421) and 0.14% (2/1421) without difference among GR groups ($P > 0.05$).

[d] These variables were submitted to a non-parametric analysis (procedure NPAR1WAY of SAS); Q1 = lower quartile; Q3 = upper quartile.

Table 3
Fertility results of gilts according to their backfat thickness (BF) at first mating (LSmeans ± SEM).

	Backfat thickness groups, mm		
	BFI (10–15)	BFII (16–17)	BFIII (18–23)
Return to estrus rate, % (*n*/*n*)	6.9 (28/405)	5.6 (36/649)	6.5 (24/367)
Farrowing rate, % (*n*/*n*)	90.4 (366/405)	92.6 (601/649)	92.1 (338/367)
Adjusted farrowing rate[c], % (*n*/*n*)	92.0 (366/398)	93.8 (601/641)	92.6 (338/365)
Total born piglets, *n*	12.2 ± 0.15 a	12.7 ± 0.12 b	12.4 ± 0.16 ab
Born alive piglets, *n*	10.8 ± 0.16 a	11.4 ± 0.12 b	11.2 ± 0.16 ab
Total stillborn[d], % (Q1–Median–Q3)	7.4 ± 0.76 (0–0–9.1)	6.2 ± 0.45 (0–0–8.7)	6.5 ± 0.66 (0–0–8.3)
Intra-partum stillborn[d], % (Q1–Median–Q3)	6.3 ± 0.70 (0–0–8.3)	5.2 ± 0.41 (0–0–7.7)	5.4 ± 0.61 (0–0–7.7)

a,b: Different letters indicate statistical differences in the same row ($P < 0.05$).

[c] Calculated by excluding dead females and females removed for non-reproductive reasons. Overall abortion and failure to farrow rates were, respectively, 0.63% (9/1421) and 0.14% (2/1421) without difference among GR groups ($P > 0.05$).

[d] These variables were submitted to a non-parametric analysis (procedure NPAR1WAY of SAS); Q1 = lower quartile; Q3 = upper quartile.

Table 4
Characteristics of gilts at farrowing and of their litters according to the growth rate (GR) between birth and first mating (LSmeans ± SEM).

	Growth rate groups, g/d		
	GRI (600–700)	GRII (701–770)	GRIII (771–870)
Females and litters weighed at farrowing	273	539	282
Weight at farrowing, kg	196.0 ± 0.77 a	206.7 ± 0.55 b	217.0 ± 0.76 c
Backfat thickness at farrowing, mm	16.6 ± 0.17 a	17.0 ± 0.12 ab	17.3 ± 0.16 b
Litter weight, kg	16.6 ± 0.22	17.0 ± 0.16	17.1 ± 0.21
BW[d] of born alive and stillborn piglets, kg	1.46 ± 0.01	1.44 ± 0.01	1.42 ± 0.01
BW[d] of born alive piglets, kg	1.46 ± 0.01	1.45 ± 0.01	1.42 ± 0.01
CV[e] for BW of born alive and stillborns, %	15.9 ± 0.38 a	17.1 ± 0.27 b	18.1 ± 0.38 b
CV[e] for BW of born alive piglets, %	15.3 ± 0.38 a	16.5 ± 0.27 b	17.4 ± 0.37 b
Piglets with weight <1200 g	2.5 ± 0.18 a	2.8 ± 0.12 ab	3.1 ± 0.17 b
Litters with CV for BW > 20%, *n* (%)	66 (24.2) a	156 (28.9) a	102 (36.2) b

abc: Different letters indicate statistical difference in the same row ($P < 0.05$).

[d] Birth weight.

[e] Coefficient of variation.

($P < 0.05$) compared to GRI and GRII groups (Table 4). There were no differences among BF groups ($P > 0.05$) in CV of birth weight, number of piglets with less than 1,200 g and in percentage of litters with a CV for birth weight > 20% (Table 5).

Body weight at farrowing differed among GR and BF groups (Tables 4 and 5). Greater BF at farrowing was observed in GRIII than in GRI group (Table 4) whereas BF at farrowing was different among the three BF groups (Table 5). Body weight and BF, at mating and at farrowing, respectively, did show only a slight correlation ($r = 0.21$; $P < 0.0001$ and $r = 0.32$; $P < 0.0001$, respectively).

Overall, more stillborns (8.3% vs. 6.4%) and heavier piglets (1,529 g vs. 1,425 g) were present in gestations >115 d than in those ≤115 d ($P < 0.05$). A greater percentage ($P < 0.05$) of sows with gestations >115 was observed in GIII (15.3%) than in GI (8.6%) and GII (10.9%) groups. Only in GRIII group, gestations >115 d resulted in greater percentage of sows with stillborns (65% compared to 48%) and

Table 5
Characteristics of gilts at farrowing and of their litters according to the backfat thickness (BF) at first mating (LSmeans ± SEM).

	Backfat thickness groups, mm		
	BFI (10–15)	BFII (16–17)	BFIII (18–23)
Females and litters weighed at farrowing	291	516	287
Weight at farrowing, kg	203.5 ± 0.85 a	206.3 ± 0.64 b	210.7 ± 0.86 c
Backfat thickness at farrowing, mm	16.1 ± 0.16 a	16.8 ± 0.12 b	18.2 ± 0.16 c
Litter weight, kg	16.8 ± 0.21	17.1 ± 0.16	16.8 ± 0.21
BW[d] of born alive and stillborn piglets, kg	1.45 ± 0.01	1.44 ± 0.01	1.43 ± 0.01
BW[d] of born alive piglets, kg	1.45 ± 0.01	1.45 ± 0.01	1.44 ± 0.01
CV[e] for BW of born alive and stillborn, %	16.5 ± 0.37	17.1 ± 0.28	17.4 ± 0.38
CV[e] for BW of born alive piglets, %	15.9 ± 0.37	16.5 ± 0.28	16.9 ± 0.37
Piglets with weight <1200 g	2.7 ± 0.17	2.8 ± 0.13	2.8 ± 0.17
Litters with CV for BW > 20%, *n* (%)	86 (29.6)	148 (28.7)	90 (31.4)

abc: Different letters indicate statistical difference in the same row ($P < 0.05$).

[d] Birth weight.

[e] Coefficient of variation.

greater percentage of stillborn piglets (10.9% compared to 8.3%) than in shorter gestations (≤115 d).

4. Discussion

Although farrowing rate and return to estrus rate were not influenced by GR or BF groups, litter size was increased in females with a GR > 700 g/d (GRII and GRIII) and in those with an intermediate BF (BFII; 16–17 mm). The greater number of total born piglets observed in GRII and GIII females corroborates the results of previous studies in which greater GR resulted in larger first litters (Tummaruk et al., 2000; Kummer et al., 2006; Young et al., 2008) or larger litter size over the first three parities (Tummaruk et al., 2007). The greater litter size observed in BFII group (16–17 mm) compared to BFI group (10–15 mm) agrees with the report that a greater BF at the first estrus result in more piglets born (Tummaruk et al., 2007) although a clear impact of BF on the first litter size (Tummaruk et al., 2001) or on the total born piglets over three parities (Williams et al., 2005) has not been reported in other studies. The fact that greater BF females (18–23 mm) had no increase in total born piglets compared to the least BF females (10–15 mm) suggests that BF is less critical than GR for the litter size. The slight correlation between BW and BF at first mating observed in this study ($r = 0.21$) is in agreement with previous reports of low significant correlation coefficients ($r \leq 0.31$) between BF and BW, for gilts with about 178 d (Tummaruk et al., 2007) or 230 d (Tummaruk et al., 2009) of age. The pattern of increase in lean body mass differs from that of changes in BF because increases in fatness are transient and any residual effects disappear by weaning of the first litter (Gill, 2006). Collectively, these findings strongly support the fact that BF has a less consistent effect on gilt productivity reinforcing the assumption that fatness at first mating is less important than a target weight for lifetime productivity (Foxcroft et al., 2005).

Females with rapid growth have greater concentrations of circulating IGF-1 and insulin (Cox, 1997), which can influence ovulation rate by reducing the chance of follicular atresia (Britt et al., 1988). Indeed, ovulation rate was greater (King, 1989) in heavy (101 kg) compared to light (71 kg) gilts at the onset of puberty stimulation. However, in the study of Kummer et al. (2009) no effect was observed on ovulation rate according to growth rate (626 compared to 737 g/d) at approximately 144 days of age. The concentration of progesterone, which is important for the maintenance of pregnancy and embryonic survival (Jindal et al., 1996; Van den Brand et al., 2000), is greater in females with a greater ovulation rate (Clowes et al., 1994). Thus, the larger litter size observed in fast growing gilts may be explained by a greater ovulation rate or a greater embryonic survival, or both, as it is suggested by the results of the present study.

Despite the higher number of total born piglets, GRIII gilts had more stillborn piglets compared to GRII and GRI gilts confirming previous reports that gilts with GR > 700 g/d (Kummer et al., 2006) or >860 g/d (Young et al., 2008) had more stillborns than lesser GR gilts. One reason for the number of stillborns in greater GR gilts (GRIII) can be related to their overweight (Dial et al., 1992) at farrowing. Although females of all GR groups were on average above the recommended body weight of about 180 kg at farrowing (Williams et al., 2005), the percentages of stillborns in GI (5.5%) and GII (6.1%) groups were below 7%, which is the maximum acceptable number of stillbirths (Dial et al., 1992; Muirhead and Alexander, 2000). Obstruction of the birth canal may result in difficulty in delivery of piglets, prolonging the farrowing. In the present study, farrowing length was not measured but there is evidence that excessive fat contribute to the obstruction of the birth canal (Ash, 1986) and that heavy females can have a prolonged parturition and consequent increase in stillborns due to a less stretched fetal canal and weak uterine contractions (Bos, 1987).

Another possible reason for the increase of intra-partum stillborns is the variation in birth weight. Overall, the average CV of birth weight was 17%, which is within the range of 16–24% reported for litters obtained with Large White × Landrace crossbred sows of parity 1–6 (Quiniou et al., 2002). The greater CV for birth weight and greater percentage of litters with a CV of birth weight > 20% observed in gilts of GRIII (i.e. >770 g/d) group probably contributed to their greater stillbirth rate. It is known that increasing litter size is linked to the decrease of birth weight and increase in stillbirth rate (Dial et al., 1992; Borges et al., 2005; Baxter et al., 2008). Lesser birth weight results in less viability and increased risk of asphyxia (Sprecher et al., 1974; Ash, 1986), hence greater intra-partum death. The greater number of piglets weighing <1,200 g observed in GRIII than in GRI gilts probably contributed to stillbirth since the overall occurrence of stillborns was greater ($P < 0.05$) in piglets weighing <1,200 g than in heavier ones (9.6% compared to 5.2%). Furthermore, stillborn weighed less (1305 ± 10.9 g) than born alive (1420 ± 2.8 g) piglets ($P < 0.05$) as previously shown (Le Cozler et al., 2002; Baxter et al., 2008).

Stillbirth can also be associated with a long gestation period (Dial et al., 1992), which was confirmed in the present study, for females of the GIII group with a gestation length > 115 d. Secondary uterine inertia may occur in a farrowing complicated by delivery of large piglets or delivery of large litters (Ash, 1986). Indeed, in GRIII group, intra-partum stillborn piglets derived from sows with a gestation > 115 d were heavier (1468 g compared to 1359 g) than those of gestations ≤115 d. The delivery of a high number (12.8) of relatively large piglets probably accounted for the higher stillbirth rate (10.9%) observed in long gestation GRIII sows compared with short gestations (8.3%). Therefore, greater stillbirth rate in GRIII can be explained by the delivery of large litters and low birth weight piglets as well as by the delivery of large piglets in sows with gestations >115 d.

5. Conclusions

The litter size of gilts increases when GR exceeds 700 g/d and when BF is of 16–17 mm. However, the stillbirth rate increases in gilts with a GR > 770 g/d. Considering the feed costs during the non-productive period, from selection to first pregnancy, the number of born alive and the uniformity of birth weight observed in the present study, it seems advantageous to perform the first mating of gilts, irrespective of age, provided gilts have a GR between 600 and 770 g/d and a BF ≥ 16 mm.

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Capability of frozen–thawed boar spermatozoa to sustain pre-implantational embryo development

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ABSTRACT

The present study was designed to evaluate the competence of frozen–thawed (FT) boar spermatozoa on the developmental ability of early porcine embryos under *in vitro* and *in vivo* conditions. Repeat deep uterine insemination was applied to sows ($n = 12$) at 30 h and 36 h after estrus detection, using either 750×10^6 of liquid or FT motile spermatozoa in a volume of 5 mL. Semen was pooled from mature Pietrain boars ($n = 3$) of proven fertility and classified as “good sperm freezers” in previous experiments. Only sows with preovulatory follicles identified during the first insemination, and those that had ovulated 6 h after the second insemination were used in the experiment. Sows were subjected to laparotomy on Day 2 of the estrous cycle (the onset of estrus classified as Day 0), and only one oviduct of each animal was flushed. The collected embryos (zygotes and two to four cell embryos) were cultured in vitro for 96 h. Embryos from the contralateral oviduct were permitted to develop *in vivo* for the same period of time. Fertilization rates were 94.4% and 90.9% for liquid ($n = 90$) and FT ($n = 77$) insemination groups, respectively, and did not differ significantly between groups. The use of FT semen for insemination did not affect embryo development and embryo quality in terms of total cells number per embryo. In contrast, these parameters were affected by the culture system ($P < 0.001$). These data indicate that when an optimal protocol for insemination with FT semen is used, normal fertilization rates, embryonic development, and embryo quality are obtained, and consequently acceptable farrowing rates and prolificacy can be expected.

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1. Introduction

There has been an increased development and improvement of emerging reproductive technologies in the pig industry in recent years. Sperm cryopreservation, which contributes to the conservation of porcine genetic resources and to the introduction of new genetics into the porcine breeding population is one of these technologies. Frozen–thawed (FT) semen is a useful alternative to the transportation of live boars and allows large numbers of females to be inseminated over extended periods. Despite the growing interest, the commercial application of FT semen at the beginning of the new millennium was restricted to 1% of the porcine artificial insemination worldwide (Wagner and Thibier, 2000). Reduced fertility and litter size achieved when FT semen is used under field conditions, as compared with those obtained with liquid semen, discouraged its use (Johnson et al., 2000; Martinez et al., 2005; Roca et al., 2006a,b). Increased fertility rates have been reported using improvements in cryopreservation protocols (Bussiere et al., 2000; Eriksson et al., 2002) and new procedures for artificial insemination (AI), such

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as the deep intrauterine insemination (DUI; Martinez et al., 2002), which allows a decrease in the minimal number of FT spermatozoa, whilst maintaining acceptable fertility (Roca et al., 2003). However, the performance of FT spermatozoa is still highly variable. This variability has been associated with factors including the number of AIs performed, insemination dose, insemination–ovulation interval, and post-thaw sperm quality.

It is evident that cryopreservation alters sperm motility and membrane status (revised in Watson, 2000) resulting in a shorter sperm lifespan (Waberski et al., 1994) and consequently the interval between AI and ovulation is considered as one of the main factors affecting reproductive performance when FT semen is used. In addition, it has been reported that cryopreservation induces DNA damage in boar spermatozoa (Fraser and Strzezek, 2005, 2007; Jiang et al., 2007; Hu et al., 2008; Fraser et al., 2009) that could be associated with an altered capacity for normal fertilization and/or embryo development, and might be linked to early embryo death, as suggested in human studies (Agarwal and Said, 2005). Hence, the process of freeze-thawing spermatozoa may adversely affect both farrowing rate and litter size. Although farrowing rate and litter size are mainly conditioned by fertilization rates and early embryo development, the ability of FT boar spermatozoa to sustain pre-implantational embryonic development has not been reported.

The objective of the present study was to examine the influence of FT spermatozoa on developmental ability of porcine embryos cultured under *in vitro* or *in vivo* conditions.

2. Materials and methods

2.1. Animals

The experimental protocols were reviewed and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain.

Weaned crossbred (Landrace × Large White) sows (from two to six parities) were randomly selected for the experiment. Sows were individually held in crates, in a mechanically ventilated confinement facility under commercial production conditions (Agropor S.L, Murcia, Spain). Sows were fed a commercial ration twice a day and water was provided *ad libitum*.

2.2. Detection of estrus and ovarian status

Estrous detection was carried out twice a day, beginning 2 days after weaning, by exposing females to a mature boar and applying manual back-pressure. Females that showed a standing estrous reflex were considered to be in heat. Only sows that exhibited first standing reflex on the afternoon of the 4th–5th day after weaning were used in this experiment.

Ovarian status of the sows exhibiting a standing reflex was examined by transrectal ultrasonography (Pie Medical SC100 Scanner, Maastrich, The Netherlands), using a 7.5 MHz multiple scan angle transducer as described by Soede et al. (1992) and modified by Bolarin et al. (2009a), in order to assess the time of ovulation. The ovaries were scanned four times: 12 h after onset of oestrus, just before each of the two inseminations and 6 h after second insemination. Both ovaries were checked simultaneously for functional structures such as preovulatory follicles, corpora hemorrhagica, and corpora lutea. Only sows with preovulatory follicles in the ovaries at the time of the first insemination and that had ovulated at the time of the last scan were used for the experiment. This schedule ensured that at least one insemination was performed close to ovulation.

2.3. Semen processing and deep intrauterine insemination (DUI)

Inseminations were carried out with pooled semen from three mature Pietrain boars of proven fertility and classified as “good sperm freezers” (Roca et al., 2006a) in previous experiments (Bolarin et al., 2009b). After collection, seminal characteristics (subjective sperm motility and acrosome integrity) were evaluated, using standard laboratory techniques, and only ejaculates with more than 75% motile spermatozoa and more than 80% intact acrosomal ridges were used.

For FT insemination doses, sperm-rich fractions from each ejaculate were extended (1:1 (v/v) in Beltsville Thaw Solution (BTS); Pursel and Johnson, 1975). The samples were frozen following the protocol used in our laboratory (Hernandez et al., 2007). Briefly, semen samples were refrigerated at 17 °C for 3 h and centrifuged at 2400 × *g* for 3 min. The supernatant was removed, and the sperm pellet was re-suspended in a lactose-egg yolk extender (LEY: 80 mL [80% (v/v) 310 mM] β-lactose and 20 mL egg yolk) to a concentration of 1.5×10^9 sperm/mL. The sperm suspension was slowly cooled to 5 °C for 2 h. The sperm suspension was then re-extended (2:1 (v/v)) with LEY containing 9% glycerol and 1.5% Orvus Es Paste. The spermatozoa were then packaged in 0.5 mL straws. The straws were frozen using a computerized freezing machine (IceCube, 1810, Minitüb, Tiefenbach, Germany) at a rate of −6 °C/min from 5 °C to −5 °C. The temperature was then lowered at −40 °C/min from −5 °C to −80 °C. Finally, the temperature was lowered at −70 °C/min from −80 °C to −150 °C. The frozen straws were stored in liquid nitrogen until they were used for AI. During each AI trial, two straws from each of the three boars were thawed by direct plunging into a 37 °C water bath. They were then shaken vigorously for 20 s. Straws were emptied and contents mixed into pre-warmed (37 °C) tubes containing BTS to obtain the required insemination doses. The suspensions were then stored at 21–23 °C until the inseminations were performed (within 1 h of thawing). The thawed sperm suspensions were evaluated for motility (Sperm Class Analyzer; Microptic, Barcelona, Spain) and plasma and acrosomal membrane integrity following the procedure described by Hernandez et al. (2007). Only FT insemination doses with sperm motility and membrane integrity ≥50% was used in this experiment.

For liquid insemination doses, sperm-rich ejaculate fractions were collected on the same day of AI, diluted in BTS to a concentration of 2×10^8 sperm/mL and kept for a maximum of 24 h at 17 °C. Each dose was a mixture of semen from the same three boars that provided the FT semen doses. Both types of insemination doses, liquid and FT, were adjusted to 750 × 10^6 motile spermatozoa/dose.

Sows were double inseminated using deep intrauterine insemination into a uterine horn at 30 h and 36 h after detection of estrus. The DUI took place for each sow in the gestation crates and was performed without sedation according to the procedure described by Martinez et al. (2002). Prior to its insertion, the DUI catheter was rinsed with warm (30 °C) BTS, refilled with approximately 2 mL of BTS and inserted forward along one uterine horn. Thereafter, liquid or FT insemination doses, in a volume of 5 mL, were slowly infused into the uterine horn through the device. Subsequently, before removing the device, additional 2 mL of BTS fluid were flushed to force any remaining spermatozoa out of the device.

2.4. *Embryo collection*

Sows were subjected to laparotomy, as described by Martinez et al. (2004), on Day 2 of the estrous cycle (D0 = onset of estrus), and one oviduct of each animal was flushed with 10 mL of NCSU-23 medium (Petters and Wells, 1993) supplemented with 0.4% bovine serum albumin (BSA) and 10 mM HEPES (flushing medium) in order to obtain zygotes and two to four cell embryos. Embryos from the contralateral oviduct were collected 4 days later (Day 6) by flushing the corresponding uterine horn with 30 mL of flushing medium. Embryos were evaluated under a stereomicroscope at 60× to assess their developmental stage and quality. Embryos were washed three times with flushing medium, placed in Eppendorf tubes containing 1.5 mL of the same medium and transported at 39 °C in a thermostatically controlled incubator to our laboratory at the University of Murcia within 2 h of collection.

2.5. *Embryo culture*

After the collection on Day 2, embryos were washed thoroughly and individually allocated into 20 μL drops of NCSU-23 medium supplemented with 0.4% of BSA under mineral oil at 39 °C, 5% CO_2 in air for 96 h (*in vitro* cultured embryos). The embryos from the contralateral oviduct that were not flushed on Day 2 were permitted to develop *in vivo* over the same period of time (*in vivo* cultured embryos).

2.6. *Assessment of embryo development stage and total cell number*

At the end of the culture period (Day 6), *in vitro* and *in vivo* cultured embryos were evaluated by stereomicroscope at a magnification of 60× to assess their survival rate, final developmental stage, morphology and total cell number per embryo. The fertilization rates were calculated as the number of viable embryos obtained after flushing divided by the total number of embryos and oocytes collected from pregnant sows. Survival rate was defined as the ratio of viable embryos after culture to the total number of embryo cultured. Embryos with normal developmental stages corresponding to the time between the onset of estrus and evaluation were considered viable. The final developmental stage on Day 6 was considered linear for the purposes of statistical analysis and scored as described by Macháty et al. (1998) in the following classes: 1, morula; 2, early blastocyst (blastocyst with a incipient visible blastocoele); 3, full blastocyst (blastocyst with a well defined blastocoele, inner cell mass and trophpoblast totally discernible); 4, expanded blastocyst (blastocyst with overall diameter increased and zona pellucida (ZP) tinned); and 5, hatching or hatched blastocyst (blastocyst with broken ZP or without ZP). Embryo diameter (excluding the ZP) and thickness of the ZP were measured with an ocular micrometer. For evaluation of total cell number, embryos were fixed in 0.4% paraformaldehyde in PBS for 20 min at ambient temperature, washed twice with PBS containing 0.1% bovine serum albumin, mounted on a slide in 4 mL of a dilution of Glycerol–PBS (3:1 (v/v)) containing 10 mg/mL of Hoechst (H-33342), and covered with a cover slip. Samples were examined with a fluorescent microscope (Eclipse E800, Nikon, Japan) using an excitation filter of 330–380 nm for blue fluorescence. The total number of blastomere nuclei, which were stained with Hoechst and displayed blue fluorescence, was counted.

2.7. *Statistical analysis*

Statistical analyses were performed using SPSS (SPSS for Windows, 2002; SPSS Inc., Chicago, IL, USA). The recovery rate of oocytes and embryos from different groups were compared using a chi-square test. The remaining results were analyzed by ANOVA using the MIXED procedure containing the fixed effects of sperm source (liquid and FT) and culture system (*in vivo* and *in vitro*) and the random effect of donor. Fertilization and survival rates were modelled according to the binomial model of parameters as described by Fisz (1980) before analysis. When the ANOVA showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set at $P < 0.05$. Results are expressed as least squares mean ± SEM.

3. Results

Fifteen sows were selected on the day of weaning as embryo donors. Sows ($n = 3$) that exhibited no obvious signs of estrus on Days 4 and 5 post weaning were eliminated from the study. The remaining sows were inseminated with liquid ($n = 6$) or FT ($n = 6$) semen. Two sows (one from each group) were excluded from subsequent analysis as they were not witnessed to ovulate during the last ultrasound scan. In two sows (one from each group) only unfertilized oocytes were recovered by flushing of the genital tract on Days 2 and 6, determining an initial pregnancy rate of 80% for each group.

The overall mean (±SEM) number of corpora lutea identified in inseminated sows was 23.5 ± 0.51. The recovery rate of oocytes and embryos in relation to the number of corpora lutea was 88.8% and did not differ significantly between AI groups. The total number of oocytes/embryos collected on Days 2 and 6 was 167 (90 and 77 from liquid and FT semen groups, respectively). Fertilization rates were 94.4% and 90.9% for each group, respectively, and did not differ significantly. The number of one-, two- and four

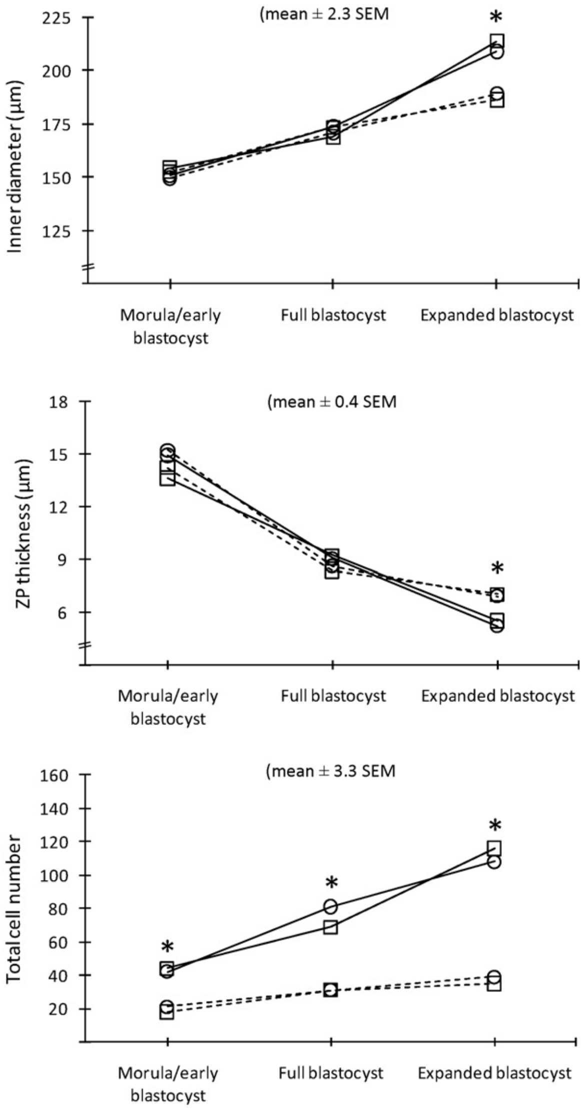

Fig. 1. Inner diameter, zona pelluzida (ZP) thickness and total cell number at different embryo development stages for embryos produced *in vivo* with liquid (□) and frozen/thawed (○) semen cultured *in vivo* (—) and *in vitro* (. . .). The use of fresh or frozen/thawed semen for insemination did not affect any of the parameters evaluated for morulae, blastocysts and expanded blastocysts obtained to the end of the *in vivo* or *in vitro* culture. The culture system (*in vivo* and *in vitro*) significantly influenced the inner diameter and the thickness of the ZP in the expanded blastocyst stage and the total cell number in all embryo stages evaluated. Asterisks indicate significantly difference ($P < 0.02$, at least) between *in vivo* and *in vitro* culture ($n = 131$ embryos; at least 11 embryos/group). The individual SEM for each group is not included due to the proximity among points.

cell embryos collected on Day 2 was similar for the two treatment groups.

The use of liquid or frozen/thawed semen for insemination did not affect any of embryo development parameters evaluated. In contrast, the embryo culture system had a significant effect ($P < 0.001$) on all parameters. The interaction between semen source and embryo culture system was not significant (Table 1).

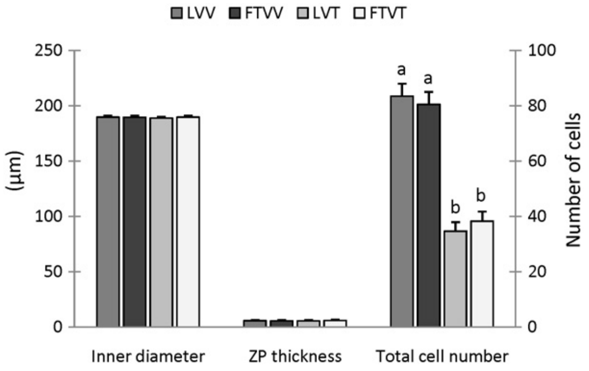

Fig. 2. Total cell number (mean ± SEM) in selected expanded blastocysts with similar size ($n = 23$). The embryos were produced *in vivo* with liquid (L) and frozen/thawed (FT) semen and cultured *in vivo* (VV) or *in vitro* (VT). *In vitro* culture conditions resulted in embryos ($n = 13$) with significantly ($P < 0.001$) lower of total cells as compared to those cultured *in vivo* ($n = 12$). ^{a,b}Means with different letters differ by $P < 0.001$.

Fig. 1 shows the characteristics of morulae/ early blastocysts, full blastocysts and expanded blastocysts produced with liquid and FT semen and cultured *in vitro* or *in vivo*. The inner diameter and the ZP thickness of morulae/early blastocysts (ranging from 149.6 ± 2.2 to 154.6 ± 2.0 µm and from 13.6 ± 1.1 to 15.2 ± 1.2 µm, respectively) and full blastocyst (ranging from 169.0 ± 2.9 to 173.9 ± 2.6 µm and from 8.3 ± 0.9 to 9.1 ± 1.1 µm, respectively) stages were similar within each stage regardless of the semen source and culture system. However, the total cell number of morulae/early blastocysts and blastocysts was significantly ($P < 0.001$) lower for embryos cultured *in vitro* (19.7 ± 3.0 and 31.1 ± 3.9, respectively) as compared with those obtained from *in vivo* culture (42.7 ± 3.0 and 73.1 ± 4.8, respectively).

The semen source did not affect the development of the embryos up to expanded blastocyst stage either. Nevertheless, expanded blastocysts developed *in vitro* had a higher ($P < 0.003$) inner diameter (187.7 ± 6.7 µm) a lower ($P < 0.02$) thickness of ZP (6.3 ± 0.5 µm) and a lower ($P < 0.001$) total number of cells (36.7 ± 7.1) than those developed *in vivo* (211.3 ± 5.8 µm, 5.4 ± 0.4 µm and 112.7 ± 5.9 µm, respectively) (Fig. 1). When expanded blastocysts with similar inner diameter and ZP thickness were selected, the differences ($P < 0.001$) in the total cell number between expanded blastocysts developed *in vitro* and *in vivo* were also evident (Fig. 2).

4. Discussion

The present study is the first to compare early development, *in vivo* and *in vitro*, of embryos produced *in vivo* with liquid and FT semen obtained from identical boars. Our results showed that the use of FT semen for insemination did not affect the percentage of viable embryos collected (fertilization rate), the survival rates, the general pattern of embryo development, and the quality of the embryos in comparison to those observed after the insemination of liquid semen.

It has been demonstrated that the optimal percentage of fertilized oocytes is reached when insemination with liq-

Table 1
Survival rates and characteristics of pig embryos produced *in vivo* with liquid or frozen/thawed semen and cultured under *in vivo* or *in vitro* conditions (LS means ± SEM).

	Culture system						
	In vivo		*In vitro*		Probability		
Semen source	Liquid	Frozen/thawed	Liquid	Frozen/thawed	Semen source	Culture system	Semen source × culture system
Number of embryos	40	34	45	36			
Survival rate (%)	96.1 ± 7.4	96.2 ± 7.9	68.5 ± 7.2	80.5 ± 7.6	NS	0.001	NS
Final stage (scale 1–5)	3.1 ± 0.6	3.0 ± 0.6	2.5 ± 0.6	2.6 ± 0.6	NS	0.001	NS
Inner diameter (μm)	183.4 ± 11.7	177.3 ± 11.7	161.5 ± 11.7	164.7 ± 11.7	NS	0.001	NS
ZP thickness (μm)	9.5 ± 2.4	10.3 ± 2.4	11.7 ± 2.4	11.3 ± 2.4	NS	0.001	NS
Total cell number	71.0 ± 4.9	78.8 ± 5.0	27.0 ± 5.2	29.5 ± 5.4	NS	0.001	NS

NS: not significant.

uid spermatozoa is performed in the interval between 12 h before to 4 h after ovulation (Waberski et al., 1994; Soede et al., 1995). Similar fertilization rates to those following AI with liquid semen have been obtained with FT semen if the interval between insemination and ovulation was at maximum 4 h (Waberski et al., 1994). In the present study, sows were inseminated twice during oestrus. Only the sows with preovulatory follicles during the first insemination and that had ovulated 6 h after second insemination were evaluated. Therefore, we can assume that at least one of the two inseminations was performed within the optimal insemination to ovulation interval. As a consequence the fertilization rate obtained in our study with FT semen (91%) was similar to that achieved with liquid semen (94%). These data confirm those reported by Waberski et al. (1994) and Bertani et al. (1997), where the insemination of FT semen was performed within the optimal interval between insemination and ovulation. Unfortunately, details of the embryo development ability were not included in the aforementioned studies. The results of the present study showed that embryos cultured *in vivo* (in the female genital tract until Day 6) had a similar survival rate, final development stage, inner diameter, ZP thickness, and total cell number regardless of the semen source. Embryo development and quality in embryos cultured *in vitro* were not influenced by the type of semen either.

Although reproductive performance of FT spermatozoa is still very variable, there have been several reports showing acceptable farrowing rates and prolificacy with FT semen when applied under field conditions (Eriksson et al., 2002; Roca et al., 2003; Bolarin et al., 2006, 2009b). In agreement with these reports, present data indicate that when the interval between AI and ovulation is appropriate, FT sperms are able to fertilize a high proportion of oocytes and produce good quality embryos and normal embryonic development.

It is known that when comparisons are made on the basis of similar numbers of motile spermatozoa, fertility results with FT semen are still generally poorer than with liquid semen, indicating that even the viable subpopulation after cryopreservation is compromised (Watson, 2000). We used the same number of motile spermatozoa (750×10^6) to prepare the liquid and FT insemination doses and no differences in any of the embryo parameters evaluated were detected. These could be due to two factors. First this study used boars with good sperm freezability. The fact that boars can be classified as “good freezers” or “bad freezers” implies that the number of surviving spermatozoa and the functional ability of the surviving sperm population after cryopreservation is extremely variable among boars (Roca et al., 2006a). Spermatozoa from “good freezers” might present certain characteristics that predispose towards survival under cryopreservation stress (Watson, 2000). We could speculate that with the boars and the cryopreservation protocol used in the present study, the damage of the post-thaw survivor sperm population was low enough to support fertilization and sustain embryo development in a comparable pattern to liquid spermatozoa. Secondly, it has been showed that many thawed spermatozoa, although motile, are unable to reach the oviduct and therefore fertilize the oocyte (Pursel et al., 1978; Holt, 2000). This suggests that defective sperm transport in the female genital tract after traditional (cervical) insemination might be an important limitation for FT spermatozoa. In our study, the sows were inseminated using the DUI procedure (Martinez et al., 2002). Data from previous reports from our laboratory (Roca et al., 2003), where the fertility was not different after DUI deposition of 1×10^9 and cervical deposition of 6×10^9 FT spermatozoa, confirm that failure of fertilization following cervical insemination may be more associated with an inadequate transport of the FT sperm within the sow genital tract than with their potential fertilizing capacity. During cervical insemination, sperm come into contact with the cervical canal and its secretions, which do not occur during DUI. The mucus-filled folds of the cervical canal may provide a difficult barrier for the passage of many spermatozoa which can be simply trapped and killed. When the semen is deposited directly into the depth of a uterine horn, a reduction in sperm number can be used because of in addition to bypass the cervical canal, semen backflow is avoided. It seems clear that under the conditions used in our study (good freezer boars, DUI and appropriate time of insemination) the fertilization rates and the quality and pattern of embryo development was not impaired by the use of FT spermatozoa.

On the other hand, our results showed differences between *in vitro* and *in vivo* embryo culture manifested through survival rate, developmental stage, embryo diameter, thickness of the ZP, and total cell number/embryo, regardless of source of semen used for insemination. In agreement with Macháty et al. (1998), embryos produced *in vivo* and cultured *in vitro* had delayed development and lower total cell number compared with those cultured *in vivo*. It could be speculated that as a consequence of the delayed embryo development, the embryos cultured *in vitro* had a lower inner diameter, a higher thickness of ZP and a lower total cell number. However, from the present results (Figs. 1 and 2) it is evident that when the embryos were grouped by their developmental stage (morulae, full blastocysts and expanded blastocysts) the total number of cells per embryo was significantly lower in embryos cultured *in vitro* in comparison with their *in vivo* counterparts, regardless of diameter and ZP thickness. The differences were increased when embryos reached the expanded blastocyst stage. These data could suggest that embryos cultured *in vitro* are able to grow and expand in a similar way to those obtained *in vivo* at the same stage, but that cellular proliferation is severely reduced during *in vitro* culture, indicating suboptimal culture conditions as previously reported for ruminant (Thompson, 1997; Rizos et al., 2002) and porcine (Macháty et al., 1998) embryos.

In conclusion, the study showed that embryos derived from FT semen have similar developmental competence and quality in terms of morphology and total number of cells per embryo than those derived from liquid semen, regardless of the culture system used (*in vivo* vs. *in vitro*). The data suggest that when an optimal protocol for insemination with FT semen is used, including the appropriate time of insemination in relation to ovulation, the use of "good freezer" boars and the deposition of insemination dose in the depth of a uterine horn, normal fertilization rates and embryonic development are obtained. Consequently, acceptable farrowing rates and prolificacy should be expected after the insemination with frozen–thawed semen.

Acknowledgments

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Involvement of opioids and catecholamines in stress modulation of LH secretion in the male pig

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ABSTRACT

The effects of different acute stressors on LH secretion and their possible interactions with opioidergic and catecholaminergic-modulation of LH secretion were investigated using gonadectomized male miniature pigs. Nose-snare (NS) or high intensity cracker blast (CB) or ACTH (1 iu/kg BW) was administered 3 h after start of blood sampling. Animals also received either naloxone (Nal; 1 mg/kg BW) or propranolol-a beta-adrenergic antagonist (Pro; 0.5 mg/kg BW) or saline. Naloxone and propranolol were given 30 (Nal) or 15 min (Pro), before the application of stressor or ACTH. Blood samples were collected every 10 min for 6 h.

Neither the acute stress of NS nor CB altered the LH secretion. ACTH increased the mean plasma LH concentrations and the LH pulse amplitude ($p \leq 0.01$) but not the pulse frequency. Naloxone elevated the mean LH values in controls, but had no effects on LH pulse frequency or pulse amplitude. Naloxone-induced increase in LH concentrations was attenuated by NS and ACTH. There was an increase in mean plasma LH values ($p \leq 0.05$), LH pulse amplitude ($p \leq 0.01$) and pulse frequency ($p \leq 0.05$) after treatment with propranolol. Nose-snare caused a reduction in the propranolol-induced increase of LH pulse frequency and pulse amplitude.

In conclusion, although, the transient painful stress of NS does not affect the LH values, it alters the opioidergic and catecholaminergic-modulation of LH secretion. The interference with opioid system is possibly mediated by ACTH.

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1. Introduction

It is now well recognized that the stress deteriorates the reproductive functions in human as well as in laboratory and farm animals. Stress not only stimulates the adrenal axis but also inhibits LH secretion and in severe cases, it can lead to an-ovulation and infertility (Rivier and Rivest, 1991; Phogat et al., 1997a,b; Dobson and Smith, 2000). Routine management practices in animal farming such as mixing with unfamiliar animals, transportation and detection of oestrus in female pigs may act as physical or psychological stress resulting in increased secretion of cortisol (Farmer et al., 1991; Dalin et al., 1993).

Treatment of sows with adrenocorticotrophic hormone (ACTH) or glucocorticoids during the follicular phase of oestrous cycle has been shown to impair the pre-ovulatory LH surge and to block the ovulation (Barb et al., 1982; Hennessy and Williamson, 1983). These in turn cause follicular cysts and silent oestrus. Both ACTH and cortisol attenuated LH pulse frequency in pre-pubertal gilts (Fonda et al., 1984). Cortisol reduced GnRH-induced LH secretion from dispersed porcine pituitary cells in vitro (Li, 1987). Furthermore, transport stress enhanced plasma cortisol concentrations and impaired the positive feed back

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of oestradiol-17β on LH release in ovariectomized pigs (Turner et al., 1999a).

The effects of stress on reproduction are dependent on time, type, intensity and duration of stress (Briski and Sylvester, 1987; Briski, 1996). Exposure to repeated daily acute but short stress of nose-snare prior to and during oestrus results in transient elevation of cortisol but did not affect reproductive performance in gilts (Turner et al., 1998). In contrast, sustained elevation of cortisol brought about by exogenous administration of ACTH impaired the LH surge, oestrus and ovulation (Turner et al., 1999a). Underlying steroid environment has been shown to influences the hypothalamo-pituitary response to stress (Briski and Sylvester, 1988).

Different kinds of stress which activate the hypothalamic–pituitary–adrenal axis, also result in activation of sympathetic–adrenal medullary axis and consequently in elevation of catecholamines in the brain and in the peripheral blood (Axelrod and Reisine, 1984; Ehrhart-Bornstein and Bornstein, 2008). Response to stress is also prominently controlled by endogenous opioids (Akil et al., 1984; Rushen and Ladewig, 1991). On the other hand, both catecholamines and opioids are well known modulators of LH secretion in different species. Interestingly, few studies indicated that opioidergic (Petraglia et al., 1986) and catecholaminergic (Higuchi et al., 1986; Martin et al., 1995) pathways are involved in mediation of stress-induced alterations in LH release in rodents. But, no information is available considering these interactions in other species including pig where opioidergic and catecholaminergic neurons are existing in GnRH neuronal vicinity (Leshin et al., 1996; Kineman et al., 1988a,b). Thus, the present study was designed to re-evaluate and further elucidate the effects of acute stress on LH secretion and specifying the pathways involved. To achieve this aim, male gonadectomized pigs were imposed to acute painful stress of nose-snare (Rushen and Ladewig, 1991) or a psychological stress of noise (Ampelas et al., 2002) with or without prior inhibition of endogenous opioids or catecholamines.

2. Materials and methods

2.1. Animals

A total of 16 mature male Göttingen-miniature-pigs (10 ± 2 months-old; 30 ± 3 kg body weight, BW) were used in this study. The animals were orchidectomized 1 month prior to commencement of experiments. We used gonadectomized animals to avoid confined results due to possible interactions of gonadal steroids with any of the treatments (Briski and Sylvester, 1988). Twenty days before start of experiments, animals were housed in individual cages allowing visual contact with each other in environmentally controlled building (temperature: 20–22 °C; light on: 0600–1800 h). To minimize the stress of handling, animals were habituated to frequent contacts with the persons who participated in experimental procedures. Pigs were provided with standard diet twice daily, 3 h before the start of the sampling and after the end of the sampling. They had access to water ad libitum at all times. Four days prior to the start of the experiment, each animal was surgically provided with an indwelling jugular catheter under general anaesthesia using the technique previously described (Parvizi and Ellendorff, 1982).

The experiment was conducted in four trials with four animals in each trial. To circumvent inter-animal variations, each animal served as its own control. In series of samplings twice a week, each animal was subjected to up to five stressor/drug combinations and the control blood sampling. A single novel stressor was applied to all animals at the same day. All other treatments were applied in a randomized manner. Due to blockade of catheters, sampling could not be completed in some pigs. The studies were performed in accordance with procedures approved by the regional Animal Ethics Committee.

2.2. Treatments

2.2.1. Stressors

Nose-snare and high intensity cracker blast were used as stressors. Further, stress was simulated by treating the animals with ACTH applications. Blood sampling was started at 0800 h. Blood samples (2 ml each) were withdrawn at 10 min intervals for a period of 360 min. Animals were never subjected to nose-snare or cracker blast before the start of the first blood sampling. Plasma samples were stored at −20 °C not longer than three weeks until assayed for LH and cortisol.

Nose-snare (NS): 180 min after the start of sampling, animals were restrained with nose-snare for two min each on four occasions at 30 min. intervals.

Cracker blast (CB): 180 min following the start of sampling, two crackers of high intensity sound (90–95 dB at 4 m distance) were blasted at three min. intervals next to the room where animals were housed so that persons and smoke due to cracker blast was not visible to them. According to the manufacturers, these crackers are not painful for the ears of an adult person at a distance of four meters.

Adrenocorticotrophic hormone: A single iv injection of ACTH (1 iu/kg BW, dissolved in 0.5 ml saline; Porcine ACTH Fragment 1-39; Sigma–Aldrich, Steinheim, Germany) was applied 180 min after the start of blood sampling.

2.2.2. Stressors in combination with neuro-modulators

Animals were subjected to treatments with either vehicle (0.5 ml saline), intra venous injection of 1 mg/kg BW naloxone (Nal, naloxone hydrochloride dehydrate; Sigma–Aldrich) or 0.5 mg/kg BW propranolol (Pro, DL-propranolol hydrochloride; Sigma–Aldrich) 30 min (Nal) or 15 min (Pro) before the application of the stressor or ACTH.

Propranolol assumed to be a potent non-selective beta-adrenergic receptor antagonist (Hoffman and Lefkowitz, 2001).

Above compounds or the vehicle were also given at corresponding time points without application of any stressor. All doses and the time of application of naloxone and propranolol were chosen according to the results obtained in previous studies (Chang et al., 1993; Tortonese, 1999; Phogat and Parvizi, 2007). Plasma cortisol levels were measured in samples withdrawn before and after applications of nose-snare and cracker blast to estimate the impact of these stressors on cortisol secretion.

2.3. Hormone analysis

Plasma LH was determined in duplicate using 100 μl of plasma by a homologous radio-immunoassay (Ponzilius et al., 1986). The sensitivity of the LH assay was 0.2 ng/ml plasma at 90% B:B_0. The intra- and inter-assay coefficients of variation were 3.5% and 6.0%, respectively. Plasma cortisol concentrations were determined by an enzyme immunoassay as described by Marc et al. (2000) and Sauerwein et al. (1991). The intra-and inter-assay coefficients of variations were 4.6% and 11.5% respectively. Plasma samples of the same animal were analyzed in the same assay to avoid the inter-assay variation.

2.4. Statistical analysis

Mean LH, LH pulse frequency and pulse amplitude before and after stress/treatment were calculated for individual animals by using computerized pulse analysis programme ‘PULSAR’ (Gitzen and Ramirez, 1987). To confirm that no LH pulse is missed by the computer programme, LH parameters were also calculated manually and were found to be the same using either of methods. The LH pulse was defined as a rise in LH greater or equal to twice of the intra-assay variance of our assay system from the preceding nadir. The pulse frequency was defined as the number of pulses in 180 min. Pulse amplitude was the difference between the maximum level during the pulse and preceding nadir. To determine the statistical significant differences SPSS (SigmaStat soft ware package) soft ware was employed. LH concentrations were analyzed by repeated-measures ANOVA followed by Tukey’s test. LH pulse amplitude and LH pulse frequency were analyzed by two-way ANOVA. Unpaired t-test was used to determine the interactions between ACTH and naloxone as well as between ACTH and propranolol by comparing the LH parameters after the respective treatments. A probability of $p \le 0.05$ was considered as statistically significant. For illustration of interactions between naloxone and nose-snare (Fig. 3) mean concentrations of plasma LH levels before applications of naloxone nose-snare and naloxone plus nose-snare were calculated as baseline and subsequent LH values in response to the treatments were calculated as percentage of this baseline.

3. Results

3.1. Stressors

Interestingly, administration of ACTH significantly increased the mean plasma LH levels ($p \le 0.01$) and pulse amplitude of LH ($p \le 0.01$) but had no effects on LH pulse frequency (Fig. 1). Neither the stress of nose-snare nor the stress of novel noise of cracker blast altered any of the three LH parameters: mean LH, mean LH pulse frequency and mean LH pulse amplitude (Fig. 1). However, exposure to both nose-snare as well as to cracker blast resulted in elevation of mean plasma cortisol values (Fig. 2). There was a fourfold increase in plasma cortisol concentrations in response to nose-snare stress ($p \le 0.001$; Fig. 2).

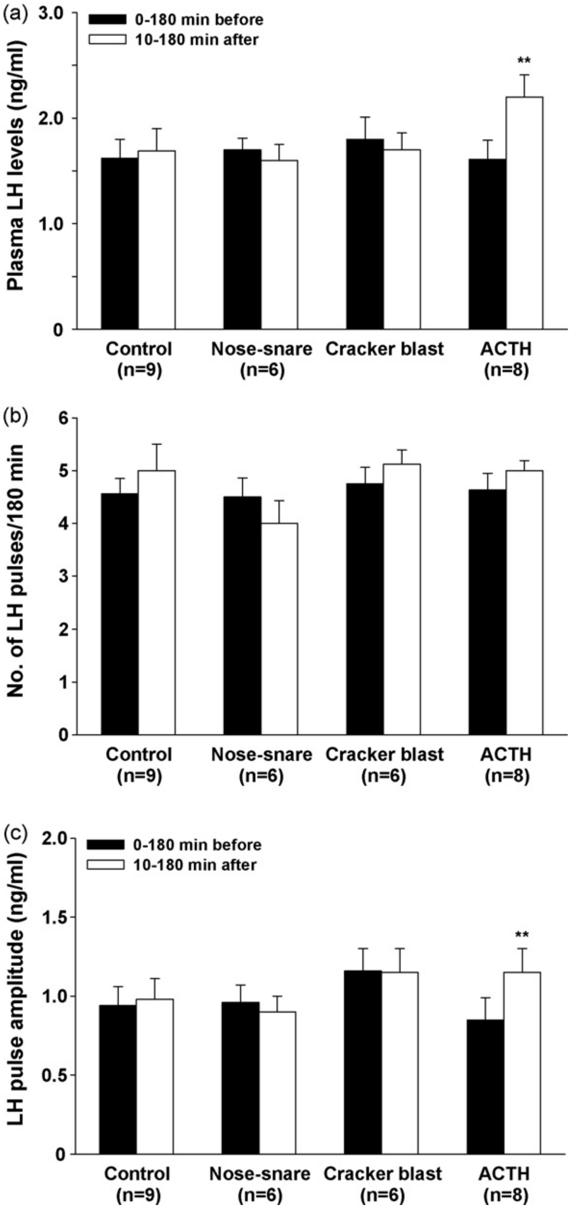

Fig. 1. Mean ± SEM plasma LH concentrations, LH pulse frequency and LH pulse amplitude in response to nose-snare (2 min each on four occasions at 30 min interval), cracker blast (two blasts at 3 min interval) and ACTH (1 iu/kg BW) in orchidectomized pigs. $^{*}p \le 0.05$; $^{**}p \le 0.01$, 0–180 min before vs. 10–180 min after treatments; number of animals are shown in parenthesis. Control received saline.

3.2. Stressors in combination with neuro-modulators

As expected, naloxone treatment caused a rise in mean plasma LH concentrations ($p < 0.01$; Figs. 3 and 4) without having any effect on the LH pulse frequency and LH pulse amplitude (data not shown). Application of nose-snare stress 30 min after naloxone administration attenuated the naloxone-induced increase in mean plasma LH levels ($p \le 0.05$; Figs. 3 and 4) leaving the LH pulse frequency and pulse amplitude unaffected (data not shown). Plasma LH concentration in response to Nal plus nose-

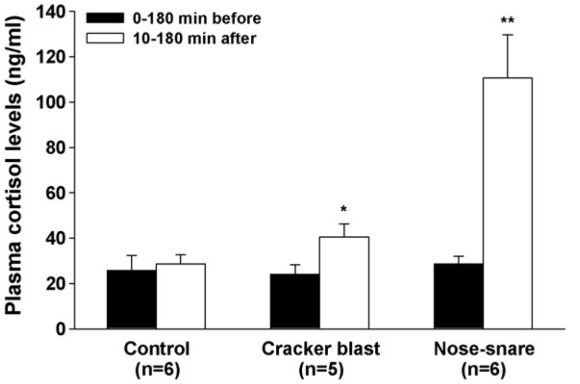

Fig. 2. Mean ± SEM plasma levels of cortisol in response to cracker blast and nose-snare in orchidectomized pigs. For more details see legend of Fig. 1.

snare reached 58.1% of LH concentrations after application of Nal alone. We also observed a significant interaction between ACTH and Nal. Adrenocorticotrophic hormone, applied 30 min before naloxone, diminished the stimulatory effect of this opioid antagonist on LH secretion (Fig. 4). Plasma LH levels in response to Nal plus ACTH treatment were significantly ($p \leq 0.05$) lower when compared to levels obtained after naloxone treatment alone (Fig. 4). The mean LH concentration after Nal plus ACTH treatment was 68.5% of concentration observed in response to the administration of naloxone alone. In contrast, there was no significant difference between LH levels after treatments with ACTH alone or Nal plus ACTH (ACTH: 2.1 ± 0.28 ng/ml vs. Nal + ACTH: 1.7 ± 0.35 ng/ml; $p = 0.13$; Fig. 4). The noise stress of cracker blast could not influence the naloxone-stimulated LH secretion (naloxone: 2.4 ± 0.15 ng/ml vs. Nal + cracker blast: 2.3 ± 0.30 ng/ml).

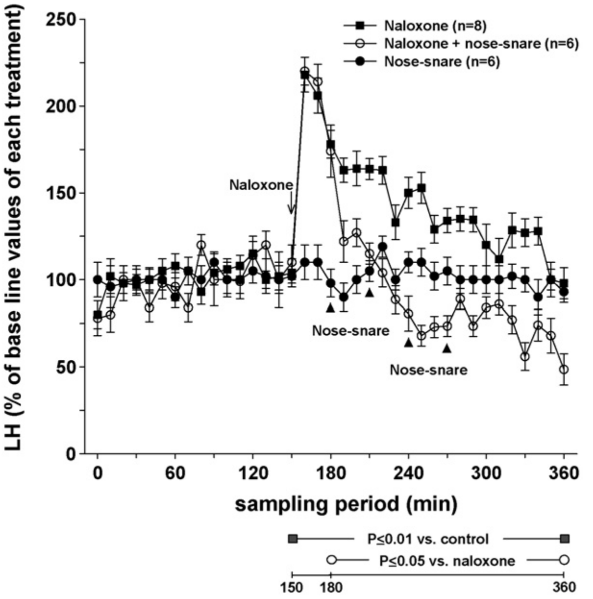

Fig. 3. LH values (percentage of baseline) in response to naloxone (1 mg/kg BW), naloxone plus nose-snare and nose-snare in orchidectomized pigs. For more details see legend of Fig. 1.

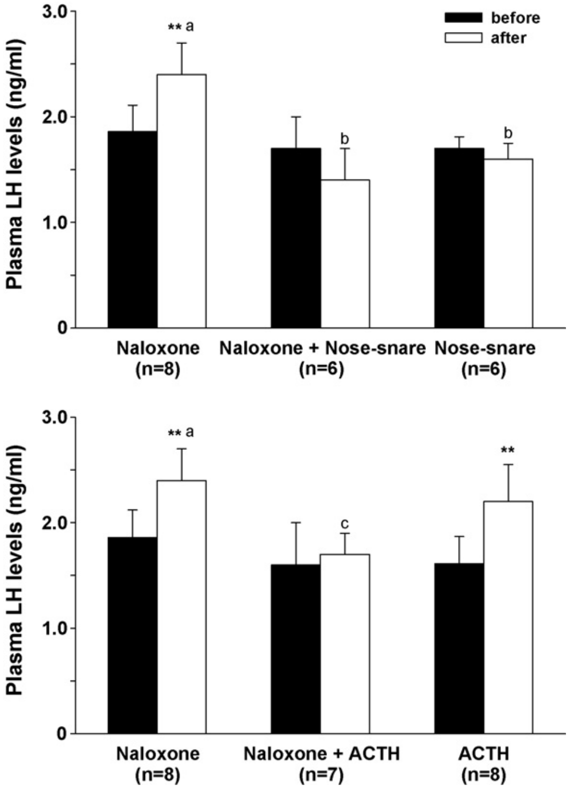

Fig. 4. Interactions between nose-snare and naloxone, and interference of naloxone with ACTH in orchidectomized pigs; a vs. b and a vs. c = $p \leq 0.05$; naloxone was applied 30 min before nose-snare or ACTH; for more details see legend of Fig. 1.

Propranolol treatment caused an elevation of all three LH parameters: mean plasma LH ($p \leq 0.05$) concentration, LH pulse frequency ($p < 0.05$) and LH pulse amplitude ($p < 0.01$; Fig. 5). Only the painful stress of nose-snare interfered with the beta-antagonist action on LH release. The effect of nose-snare was significant when the LH pulse frequency and amplitude were considered ($p < 0.05$; Fig. 5).

4. Discussion

Neither the short acute physical stress of nose-snare nor the momentary noise stress of cracker blast affected any of the three analyzed LH parameters. This cannot be attributed to the lack of intensity of the applied stress to stimulate the hypothalamic–pituitary–adrenal axis. Plasma cortisol concentrations elevated when castrated male pigs were exposed to both the nose-snare and the cracker blast. This may imply that daily repeated transient rises in the plasma cortisol do not influence the LH secretion (Turner et al., 1998). Whereas, sustained elevation of plasma cortisol concentrations over several days impaired the LH surge, oestrus and the ovulation in intact female pigs (Turner et al., 1999a) and inhibited the LH secretion in ovariectomized gilts (Turner et al., 1999b). Daily repeated intermittent but inescapable applications of electroshocks delayed the onset of puberty and reduced the mean plasma LH concentrations in gilts (Pedersen

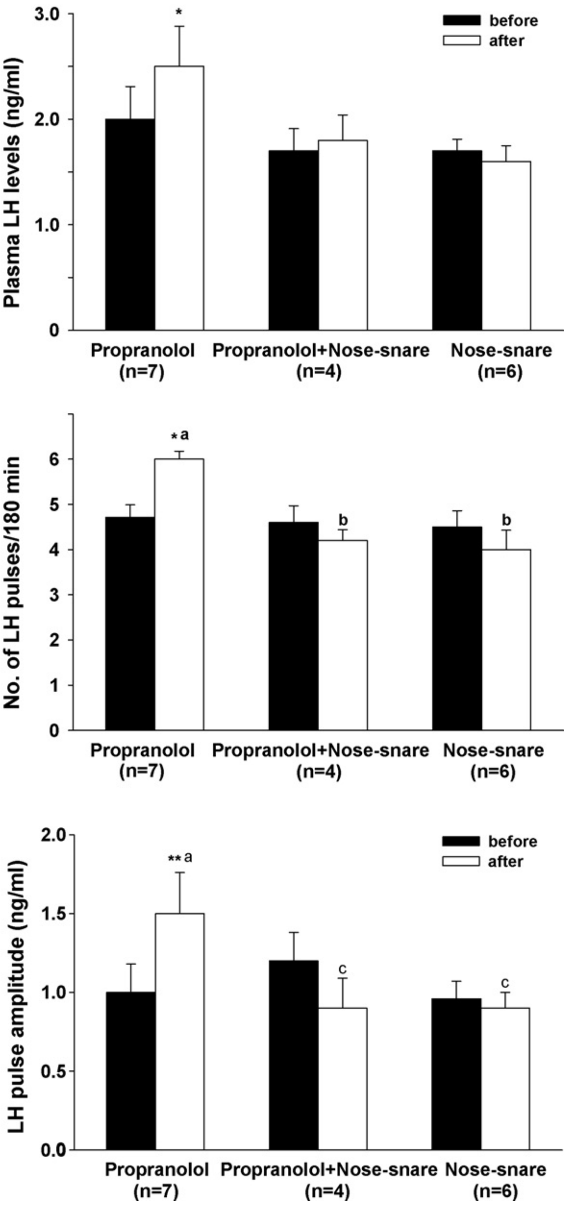

Fig. 5. LH concentrations (mean ± SEM), LH pulse frequency and LH pulse amplitude in response to propranolol (0.5 mg/kg BW), propranolol plus nose-snare in orchidectomized pigs; a vs. b and a vs. c = $p \leq 0.05$; propranolol was applied 15 min before nose-snare. For more details see legend of Fig. 1.

et al., 1996). On the other hand, the stress of only 1 h of transport was enough to abolish the oestradiol-17β-induced LH surge in female pigs (Ziecik and Zieminska, 1995). The perception of the stress and the response of the individual to the stress is a complex process and is dependent on several factors such as gonadal steroids, sex (Tilbrook et al., 1999), as well as time, type, intensity and duration of stress (Mastorakos et al., 2006 for review).

In the present study, ACTH treatment caused an increase in mean concentrations of plasma LH and the LH pulse amplitude. This seems to be in contrast to the results published by Brandt et al. (2007). In their study repeated ACTH applications suppressed the LH values. It must be, however, considered that we used male animals, but in Brandt et al. (2007) experiment animals have been females in the first oestrus post weaning. Hence, confirming that the sex and gonadal steroids play an important role in mediating the action of ACTH on LH release. Support for this assumption also comes from the studies carried out by Van Lier et al. (1999) in castrated rams. These authors reported an enhancement of the frequency of plasma LH pulses in response to ACTH treatment in wethers (Van Lier et al., 1999). ACTH action on plasma LH concentrations and LH pulse amplitude may take place at pituitary level, because ACTH increased basal LH secretion from ovine pituitaries in vitro (Matteri et al., 1986; Phogat et al., 1997a). This effect of ACTH on LH secretion was reversed by GnRH antagonist in vitro (Matteri et al., 1986).

Naloxone, as expected (Chang et al., 1993; De Rensis and Foxcroft, 1999; Estienne et al., 2002), resulted in an unambiguous increase in LH concentration in unstressed pigs. Interestingly, the naloxone-induced LH release was attenuated by ACTH and by nose-snare. This may suggest that naloxone as well as ACTH act at least via two different pathways, an inhibitory and a stimulatory one to modulate the LH release, and that the inhibitory pathway is presumably equally sensitive to naloxone, ACTH and nose-snare stress. Although, this, common pathway could not be sufficiently activated by only one of the stimuli, combination of the two, naloxone and ACTH or naloxone and nose-snare super passes the threshold for the effective stimulation. Acute immobilization stress and intraventricular injection of CRF also suppressed naloxone-induced LH release in rats (Akema et al., 1996). It is, however, conceivable that these pathways are not solely located within the hypothalamic–pituitary–adrenal-cortex axis. The sympathetic–adrenal medullary axis could also play a crucial role in mediation of these effects and interactions. Both ACTH (Valenta et al., 1986) and naloxone (Yamauchi et al., 1997) have been shown to interact with (stress) activation of adrenal medullarly release of catecholamines.

Propranolol, when given alone stimulated the LH release in the present study. Adrenergic system is known to exert stimulatory as well as inhibitory actions on LH release (Parvizi and Ellendorff, 1982; Li, 1989; Siawrys et al., 2002). The effects are sex- and steroid-dependent (Leung et al., 1982; Parvizi and Ellendorff, 1982; Taleisnik and Sawyer, 1986). Propranolol has been shown to block the inhibitory action of epinephrine on GnRH-induced LH secretion from pig pituitaries (Li, 1989). The predominant action of beta-receptors on LH release seems to be inhibitory (Leung et al., 1982; Taleisnik and Sawyer, 1986). Further, stimulation of beta-receptors attenuates the firing rate of hypothalamic GnRH neurons and in turn the pulsatile release of LH (Leung et al., 1982; Taleisnik and Sawyer, 1986).

The stress of nose-snare inhibited the propranolol-induced increase in LH pulse frequency. This confirms that beta-receptors are involved in the control of pulsatile LH release (Al-Hamood et al., 1987). Stress can induce a release of norepinephrine (Axelrod and Reisine, 1984), the later inhibits pulsatile LH secretion when applied into the brain in gonadectomized rats (Gallo and Drouva, 1979).

It is remarkable that ACTH interfered with naloxone but not with propranolol, whereas the stress of nose-snare influenced, albeit in different ways, both noloxone and propranolol action. Thus the painful stress must have activated centres outside of hypothalamic centres mediating opioidergic and catecholaminergic actions on LH. This also supports the assumption that the interactions between the stressors and the neuro-modulators at least partly take place at the level of sympathetic–adrenal medullary level. Studies in guinea-pigs also suggest that opioids and catecholamines modulates the LH RH neurones and thus the LH secretion by independent pathways (Gore and Terasawa, 2001).

The ineffectiveness of a novel noise stress may indicate that the action of the painful stress of nose-snare on LH secretion is specific. However, it is also possible that the intensity of the noise used in this study was not strong enough to stimulate any other pathways except those modulating directly and exclusively the cortisol secretion.

In conclusion, although, a transient stress is not destructive to the release of reproductive hormones such as LH, it can alter the opioidergic and catecholaminergic-modulation of LH secretion. The apparent interference with opioid system is possibly mediated by ACTH. This action of ACTH, however, needs to be confirmed in further experiments.

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Estrous cycle-dependent activity of neutrophils in the porcine endometrium: Possible involvement of heat shock protein 27 and lactoferrin

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ABSTRACT

Neutrophil infiltration into the porcine endometrium is thought to be a specific feature during the follicular phase of the estrous cycle. To specify the localization and distribution of neutrophil granulocytes at different stages of the estrous cycle, porcine uterine samples were evaluated by immunohistochemical methods using anti-bovine lactoferrin (LF) antibody. Additionally, blood samples were collected from 30 pigs at different stages of the estrous cycle with a special focus on peri-estrous phase. Manual 100-cell differential counts were performed on routinely stained blood smears and evaluated statistically. Finally, the expression of granulocyte-colony-stimulating factor (G-CSF) and heat shock protein 27 (HSP 27), which are known to influence activation of the neutrophilic granulocytic lineage, was analyzed in porcine uteri using immunohistochemistry. Results show that LF is expressed regularly in the cytoplasm of neutrophil granulocytes. An increasing infiltration of subepithelial neutrophils was detected in the follicular phase. The highest number of intra- and subepithelial LF-positive cells was found on d 2 of the estrous cycle. Maximum level was followed by a strong decrease on d 3. Blood analysis revealed that the percentage of neutrophil granulocytes was significantly lower at d 2 (26.2 ± 11.1 %) than d 1 (42.1 ± 11 %) of the estrous cycle. HSP 27 staining was predominantly localized to luminal epithelium (LE) and glandular epithelium (GE) depending on stage of the estrous cycle. Strong immunostaining of HSP 27 is only found in LE during estrus. At d 2 of the estrous cycle, HSP 27 immunoreactivity in LE and superficial GE is reduced but moderate staining is found in deep GE. G-CSF immunostaining is uniformly not detected in endometrial cells of cyclic pigs.

In conclusion, there is a clinically relevant relationship between neutrophil count in the blood and neutrophil infiltrate in the endometrium of the pig during the estrous cycle. This association may reflect the possibility of translocation of neutrophils from the blood to the endometrium up to d 2 of the estrous cycle. Additionally, HSP 27 could be a good candidate involved in migration and/or function of neutrophils within the porcine endometrium.

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1. Introduction

Many studies have already investigated changes in the immune response within the porcine endometrium during the estrous cycle. In general, immunoglobulin-containing plasma cells are present in low numbers in porcine uterine tissues (Hussein et al., 1983) indicating that other

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immune cells may play an important interactive role in the cyclic cellular changes in both the structure and function of the endometrium. In porcine uteri, T lymphocytes, macrophages and neutrophils appear to be the prominent leukocyte cell types, which are responsible for the local cellular immune response (Bischof et al., 1994). Uterine infiltration with neutrophil granulocytes attracted more attention because post-breeding migration of neutrophils into the uterus is considered to be an important reason for sperm losses (Taylor et al., 2009a). For a better understanding of physiological role of neutrophils within endometrium, it is necessary to know their normal localization, distribution, and specific activation during the estrous cycle. Indeed, there are some studies on the distribution of neutrophil granulocytes in the uteri of cycling pigs. In cycling pigs, it appears that at the late stage of the cycle (d 18–21) there is a dramatic infiltration of neutrophils into the endometrium under the influence of estrogen (Bischof et al., 1994; Kaeoket et al., 2001, 2002). However, some conflicting results are obvious regarding the localization and distribution of neutrophils in different studies. Reasons therefore might be that conventional staining methods are not sensitive enough to identify neutrophils and antibodies used recognize different cell types, such as granulocytes, monocytes, and macrophages, respectively. To obtain more reliable results about neutrophil accumulation in the porcine endometrium, it is important to find a specific marker for neutrophil granulocytes of the pig. We focused on lactoferrin (LF), because LF, an iron-binding protein, is consistently expressed in specific granules of human and animal neutrophils and is not stained in lymphocytes and monocytes (Masson et al., 1969; Bennett and Kokocinski, 1978; Esaguy et al., 1989). Several studies in humans have shown that antibodies against LF could represent a useful tool in immunostaining of active neutrophils (Skubitz et al., 1989; Afeltra et al., 1997).

Apart from the endocrine influence it is yet not clear which local immune-regulatory factors are responsible for the cyclic recruitment of neutrophils from the blood into the porcine endometrium. In inseminated or early pregnant pigs, the effects of liquids such as seminal plasma or semen extender on cytokine expression and leukocyte abundance in the endometrium have been well characterized (O'Leary et al., 2004; Taylor et al., 2009a,b). These studies have shown that liquids may have the ability to induce the expression of chemoattractant cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1). However, most of these cytokines including GM-CSF or IL-6 are neither expressed nor up-regulated in endometrium of cyclic pigs (Chabot et al., 2004), nor known as neutrophil chemoattractants (e.g. MCP-1).

As the follicular and luteal phases of the estrous cycle are correlated with functionally different metabolic, secretory and interactive requirements to uterine cell types, we hypothesize that uterine cells will be exposed to different stress conditions in a cyclic-dependent manner. Heat shock proteins (HSPs) are highly conserved anti-apoptotic proteins whose expression is induced by different kind of stresses. Small HSPs such as HSP 27 are ATP-independent chaperons, most strongly induced in response to differentiating agents, mitogens, inflammatory cytokines, and oxidants (for review see Lanneau et al., 2008). Previously, it was shown that HSP 27 is increased by estrogen in human endometrial cells (Padwick et al., 1994). Together with the observation that HSP 27 regulates the neutrophil chemotaxis (Jog et al., 2007) we suggest that HSP 27 may be a good candidate involved in migration and/or function of neutrophils within the porcine endometrium.

This study aimed to detect and quantify neutrophils in porcine endometrium during different stages of the estrous cycle using LF as a specific neutrophil marker. In addition, the percentages of neutrophils in the blood were counted to determine whether cyclic differences in neutrophil counts exist and could be linked to neutrophil accumulation in endometrium. Finally, we aimed to identify local endometrial factors such as HSP 27 which probably regulate neutrophil infiltration.

2. Materials and methods

2.1. Animals and experimental design

A total of 13 pigs of the German Landrace were obtained from the experimental station of animal husbandry, animal breeding, and small animal breeding of the University of Hohenheim. Estrus detection was performed twice daily in the presence of a boar. The day the female pig accepts the boar for mating was determined as day (d) 0 of the estrous cycle. The cyclic and not inseminated animals were slaughtered at metestrus ($n=4$; d 2–3), diestrus ($n=3$; d 10–13), proestrus ($n=3$; d 18), and estrus ($n=3$; d 0). After slaughter, specimens of the uterus were immediately fixed either in methanol/glacial acid (2:1, 24 h) or Bouin's solution (48 h) and paraffin embedded according to standard procedures. For immunohistochemical staining procedures uterine specimens were cut into 2 μm thick serial sections and mounted on Superfrost® glass slides.

Additionally, blood samples were collected from 30 non-mated pigs at different stages of the estrous cycle. Pigs were housed and fed under the same conditions as the pigs slaughtered for immunohistochemical experiments. Estrus detection was performed repeatedly as described above and the day of first standing estrus (d 0) was recorded by experienced staff. Blood was drawn into K3 EDTA 4 ml tubes and serum 13 ml tubes (Sarstedt, Nümbrecht, Germany) from the cranial vena cava at d 0 ($n=7$), d 1 ($n=8$), d 2 ($n=5$), d 3 ($n=3$), and d 15–19 ($n=7$). Shortly after bleeding, smears for the microscopic differential leukocyte count (M-Diff) were made from K3 EDTA blood. The blood smears were stained routinely with a May-Grünwald's Giemsa stain (Merck, Darmstadt, Germany). All blood samples were checked by an automated hematology analyzer (scil Vet abc, Scil Animal Care Company, Viernheim, Germany) to exclude leukocytosis or leukopenia. Blood samples for hormone measurements were centrifuged within 1 h after bleeding, serum harvested and stored at −20 °C. Determination of oestradiol (E2) and progesterone (P4) in serum samples was performed by a commercial veterinary laboratory (Synlab, Augsburg, Germany) accredited by German

Accreditation Association. Hormone levels are expressed as mean and standard deviation (SD).

2.2. Antibodies

The following antibodies were purchased and used for immunohistochemical experiments on paraffin embedded sections: goat polyclonal anti-bovine lactoferrin (LF) antibody (Bethyl Laboratories, Inc., Montgomery, USA) diluted 1:200 in phosphate-buffered saline (PBS, pH 7.4), goat polyclonal anti-human granulocyte-colony-stimulating factor (G-CSF) antibody (sc-1318, Santa Cruz Biotechnology Inc., CA, USA) diluted 1:200 in PBS, goat polyclonal anti-human heat shock protein 27 (HSP 27) antibody (sc-1048, Santa Cruz Biotechnology Inc.) diluted 1:100 in PBS, and mouse monoclonal major basic protein antibody (Clone BMK-13, Acris, Herford, Germany) diluted 1:300 in PBS.

2.3. Immunohistochemistry

Staining procedure is described exemplarily for LF immunostaining. For all antibodies used pre-treatment steps and reagents were the same if not otherwise stated. Briefly, after deparaffinization and antigen retrieval in a microwave oven in 10 mM sodium citrate buffer at pH 6.0 (3 × 5 min at 700 W) sections were processed for immunohistochemistry. Endogenous peroxidase activity and non-specific protein binding of the sections were blocked by 10% hydrogen peroxide solution in double-distilled water for 10 min at room temperature (RT) and 10% normal rabbit serum (Dako, Hamburg, Germany) for 30 min at RT, respectively. Afterwards, sections were incubated with the primary antibody at 4 °C overnight in a humid chamber (Shandon, Frankfurt a.M., Germany). The next day, sections were treated with the biotinylated rabbit anti-goat second antibody for 30 min at RT and incubated with the Strept-ABC kit (Millipore GmbH, Schwalbach, Germany). Each incubation step was followed by 3 × 5 min rinsing with PBS. The reaction product was visualized with 3,3′-diaminobenzidine–hydrogen–peroxide reagent (Biotrend Chemicals, Köln, Germany). Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, cleared with xylene, and mounted in Entellan (Merck, Darmstadt, Germany). Immunohistochemical controls were performed by (1) replacing the primary antibody with non-immune serum, (2) omitting the secondary antibody, and finally (3) incubation with DAB solution alone to ensure specificity of staining. Mammary gland tissue served as positive control for LF and HSP 27 as recommended by the supplier.

Pictures were taken using a brightfield light microscope (DMRBE, Leica, Bensheim, Germany) and a video camera (ProgRes 3012, Kontron Instruments, Watford, UK) coupled to a computer. Light microscopic evaluation was performed using the integrated Image Processing and Analysis (IPA) program.

2.4. Statistical analysis

For quantitative analysis of LF-positive cells, areas counted were divided into intraepithelial and subepithelial zones of uterine luminal epithelium. A total of 100 cells per animal and stage were counted in three random fields at 400× magnification, and the number of LF-positive cells was expressed as a percentage of positive cells per total number of counted cells. The data are expressed as mean and standard deviation (SD). Due to quantitative differences in the LF-positive population between d 2- and d 3-stages, animals from the metestrus group were divided into two subgroups each containing two animals.

The M-Diff was based on 100 leukocytes (WBC) routinely performed by two medical technologists in the clinical laboratory. Data for lymphocyte and neutrophil count are expressed as mean and standard deviation (SD). Differences in neutrophil count between different days of the estrous cycle were examined for statistical significance using ANOVA and post hoc testing. $p < 0.05$ denoted a statistically significant difference. All analyses were performed with a statistical software package (SPSS for windows, version 11.0; SPSS Inc.).

3. Results

3.1. LF immunohistochemistry

Immunostaining of sections with LF antibody clearly demonstrates that LF is selectively localized to the cytoplasm of neutrophil granulocytes (Fig. 1a–c). No specific staining of LF is observed in the uterine epithelial cells.

To exclude staining of eosinophil granulocytes, control sections were immunostained using eosinophil major basic protein antibody, previously shown to be a specific eosinophil marker (Skubitz et al., 1989). Results showed that eosinophils are concentrated mainly in the upper parts of the stroma in the endometrium, but not in intra- or subepithelial areas (data not shown). In contrast, LF-positive cells are mostly confined to intra- and subepithelial zones of uterine luminal epithelium. Number and distribution of LF-positive cells changed markedly within these zones during the estrous cycle (Fig. 1a–c). At proestrus, LF-positive cells are found exclusively in subepithelial zone in low concentrations (Fig. 1a). The number of subepithelial LF-positive cells increases dramatically during the estrus (Fig. 1b). However, intraepithelial LF-positive cells are still barely observed. The highest number of LF-positive cells is counted at d 2 of the estrous cycle. At this cycle stage there is an extensive infiltration of neutrophils into the sub- and intraepithelial zones, forming a barrier-like defense wall along the uterine epithelium (Fig. 1c). Compared to d 2, there is a dramatic decrease in LF-positive cells at d 3 of the estrous cycle (Table 1). LF-positive cells are only sporadically distributed along the subepithelial zone of the uterine luminal epithelium (data not shown). In uteri of diestrous pigs LF-positive cells are almost never detected in both zones (data not shown). Details are summarized in Table 1.

3.2. Characterization of blood samples and M-Diff

The average concentrations of estradiol-17β (E_2) and progesterone (P4) in blood samples for the different days

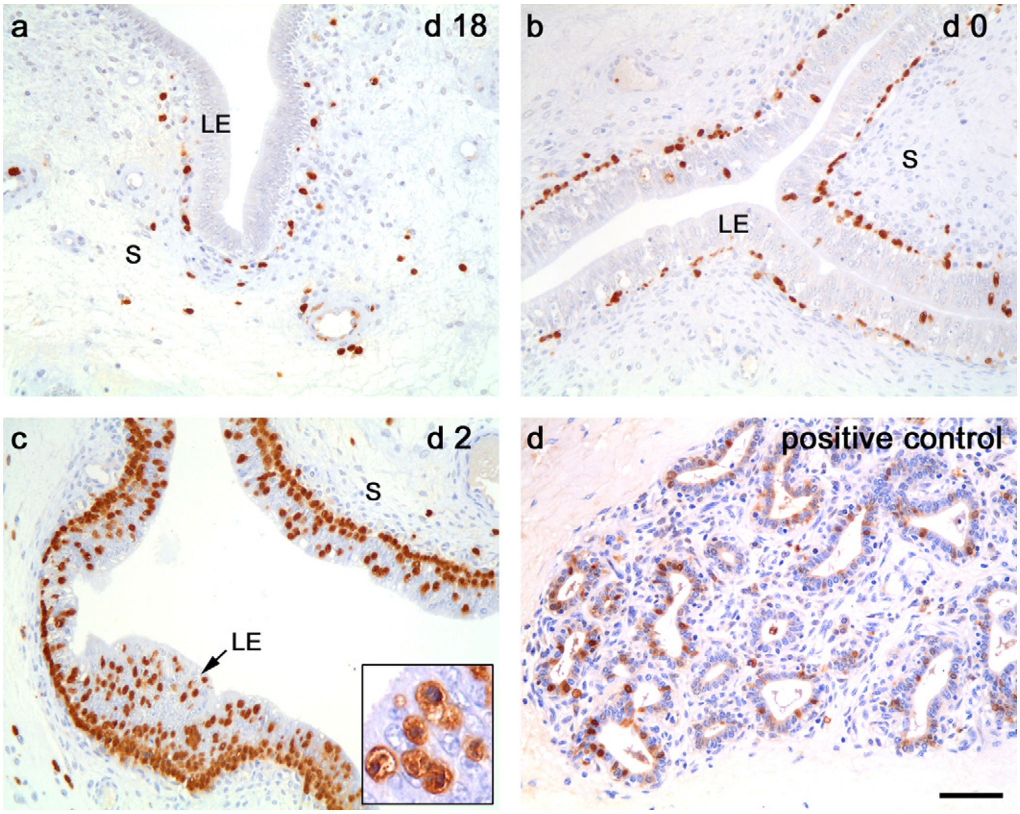

Fig. 1. Immunohistochemical distribution of lactoferrin (LF)-positive neutrophil granulocytes in porcine endometrium during the estrous cycle. Accumulation of subepithelial LF-positive neutrophils is first noticed at d 18 (a) and increased at d 0 (b). Maximum infiltration of LF-positive neutrophils in intra- and subepithelial zones of uterine luminal epithelium is present at d 2 (c). Neutrophils are characterized by segmented nuclei (c, insert). LF immunoreactivity is also found within the mammary gland epithelium showing scattered positive cells (d). LE, luminal epithelium; S, stroma of the subepithelial area. Scale bar = 120 μm.

Table 1

Intraepithelial (IE) and subepithelial (SE) number of LF-positive neutrophil granulocytes in the porcine endometrium during different days of the estrous cycle (100 cells/animal).

Day of the cycle	LF-positive cells	Animal 1	Animal 2	Animal 3	Mean value	SD
18	IE	0	0	0	0	0
	SE	1.3	3.7	15	6.7	6.9
0	IE	0.7	2	3.3	2	1.6
	SE	26.3	24	22	24.1	2.7
2	IE	25.7	52.7	–	39.2	–
	SE	27	52.7		39.9	–
3	IE	0.3	0	–	0.2	–
	SE	5.3	0		2.7	–
10–13	IE	0	0	0	0	0
	SE	0.7	0	0	0.2	0.4

of the estrous cycle are shown in Table 2. In general, concentration levels for E_2 in blood are high during follicular phase and low during luteal phase. By contrast, P4 values are very low in blood samples of d 0–1 followed by a marked increase in d 2 blood samples. P4 values are highest in blood samples of pigs obtained at d 15–16. In summary, hormone levels in the blood are within the reference ranges published for pig (Henricks et al., 1972).

Microscopic differential leukocyte counts were performed as described in material and methods. Results of lymphocyte and neutrophil counts during different days

Table 2

Concentrations of estradiol-17β (E_2) and progesterone (P4) in the blood samples of 30 pigs during different days of the estrous cycle (expressed as mean ± SD).

Day of the cycle	Number of samples (*n*)	E_2 (pg/ml)	P4 (ng/ml)
0	7	23.1 ± 14.0	0.3 ± 0.1
1	8	13.8 ± 7.9	0.4 ± 0.2
2	5	20.2 ± 7.4	2.2 ± 0.8
3	3	26.4 ± 20.8	4.8 ± 3.1
15–16	3	6.7 ± 2.0	15.5 ± 4.8
18–19	4	16.2 ± 8.9	0.5 ± 0.3

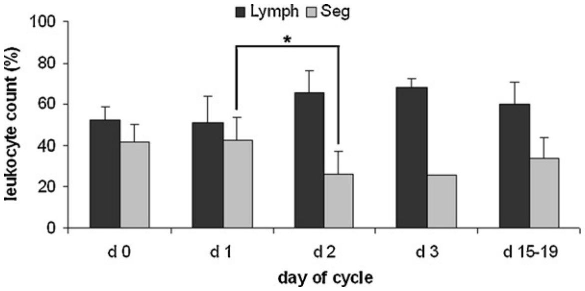

Fig. 2. Percentage of lymphocyte (Lymph) and neutrophil (Seg) counts from blood of 30 pigs collected during different days of the estrous cycle. Data are expressed as mean and SD. $^{*}p<0.05$.

of the estrous cycle are shown in Fig. 2. Statistical analysis revealed that the percentage of neutrophil granulocytes is significantly ($p<0.046$) lower at d 2 (26.2 ± 11.1%) compared to d 1 (42.1 ± 11.0%) of the estrous cycle. There are no statistically significant differences in neutrophil counts between the other days ($p>0.05$).

3.3. HSP 27 and G-CSF immunohistochemistry

HSP 27 staining is predominantly localized to luminal epithelium (LE) and glandular epithelium (GE). Immunoreactivity of HSP 27 is also observed in smooth muscle cells and endothelial cells. In all cases, staining is cytoplasmic but differential staining intensity of HSP 27 is evident depending on stage of the estrous cycle (Fig. 3a–c). During proestrus, HSP 27 protein is stained moderately in the cytoplasm of LE and GE (Fig. 3a). In contrast, strong immunostaining of HSP 27 is found in LE at estrus, whereas staining is faint or absent in GE (Fig. 3b). Interestingly, HSP 27 immunostaining is particularly strong in smooth muscle cells and endothelial cells at this time point of the estrous cycle (Fig. 3b). At d 2 of the estrous cycle, HSP 27 immunoreactivity in LE and superficial GE is reduced but moderate staining is found in deep GE (Fig. 3c). Generally, levels of immunoreactive HSP 27 decrease markedly in the cytoplasm of LE during diestrus compared to estrus (data not shown). Intense staining of HSP 27 is only detected in apical plasma membrane of LE. Low level of immunoreactive HSP 27 is concentrated in apical and basolateral compartment of GE (data not shown).

G-CSF immunostaining is uniformly not detected in endometrial cells of cyclic pigs (data not shown).

The absence of detectable staining in tissue elements in the control sections verified the specificity of the applied antibodies (data not shown). Additionally, sections of mammary gland were stained in parallel as positive controls. As clearly demonstrated in Fig. 1d, LF

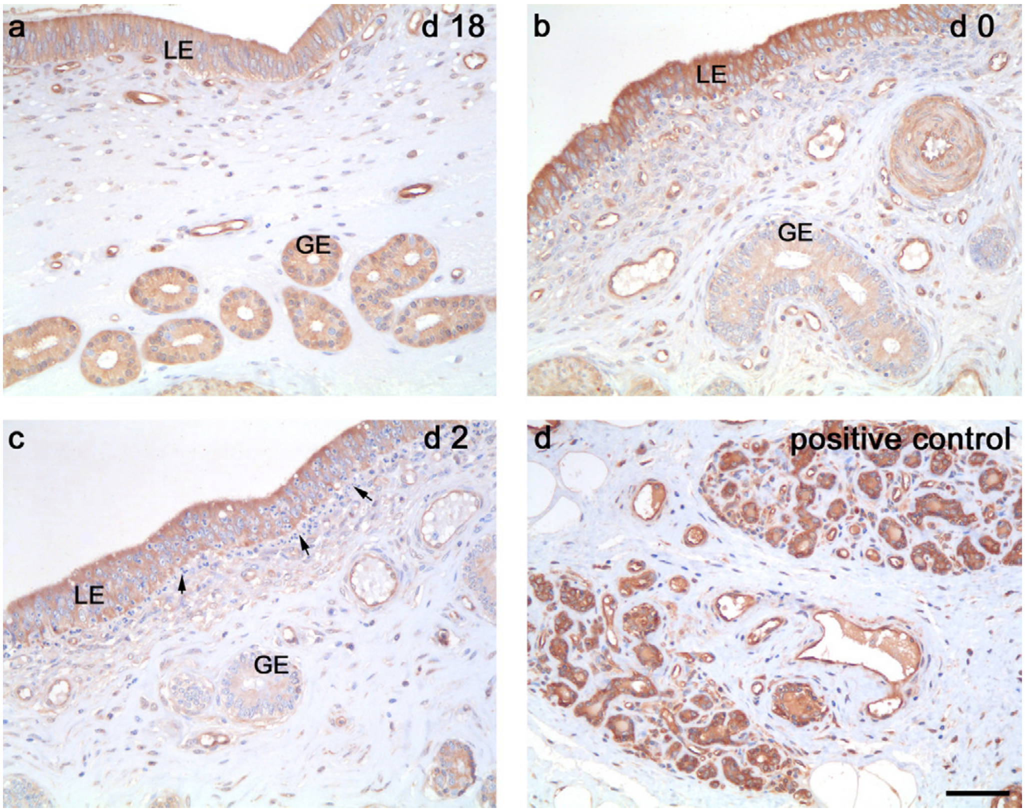

Fig. 3. Immunohistochemical localization of heat shock protein 27 (HSP 27) in luminal (LE) and glandular (GE) epithelium of the porcine endometrium during the estrous cycle. HSP 27 is stained moderately in the cytoplasm of LE and GE at d 18 (a). Strong immunostaining of HSP 27 is found in LE at estrus, whereas staining is faint in GE (b). At d 2 of the estrous cycle, HSP 27 immunoreactivity in LE and superficial GE is markedly reduced. Arrows indicate subepithelial neutrophil accumulation (c). Strong immunolabelling of HSP 27 is detected in mammary duct lobular unit (d). Scale bar = 120 μm.

immunoreactivity is found within the epithelium showing scattered positive cells. In contrast, strong immunolabelling of HSP 27 is detected in terminal duct lobular unit (Fig. 3d).

4. Discussion

The number of available spermatozoa in the porcine genital tract is rapidly and dramatically reduced within only 3–4 h after insemination (Matthijs et al., 2003; Taylor et al., 2009a). This rapid decline is indicative of an efficient clearing system consisting of backflow and phagocytosis by polymorphonuclear (PMN) leukocytes (Matthijs et al., 2003; Taylor et al., 2009a). Neutrophil granulocytes account for approximately 80 percent of intra-luminal leukocytes in inseminated pigs and they are considered to be primarily involved in clearing sperm from the uterine lumen (Taylor et al., 2009a). The prerequisites for uterine phagocytosis and defense events after insemination are mobilization and migration of neutrophil granulocytes into the stroma and epithelium of the endometrium in an estrous cycle-dependent manner. Using LF antibody we were able to localize LF protein specifically in the cytoplasm of neutrophil granulocytes. No specific staining of LF was observed in other immune cells and uterine epithelial cells, respectively. This is in good agreement with results published previously (Masson et al., 1969; Bennett and Kokocinski, 1978; Wang et al., 1997) and is a further evidence for the specificity of the applied antibody. The number and distribution of LF-positive neutrophils in the porcine endometrium changed considerably during the estrous cycle. Accumulation of LF-positive neutrophils was first noticed at proestrus, increased at estrus, and peaked at d 2 corresponding to the metestrous stage. While the increase of neutrophils in the endometrium at estrus was still recognized previously (Bischof et al., 1994), maximum infiltration of intra- and subepithelial zones of uterine luminal epithelium at d 2 is definitely a new finding. Immediately after d 2, number of LF-positive neutrophils decreased dramatically and disappeared almost completely in luteal phase. In addition, we have shown that the number of neutrophils in the endometrium correlated with neutrophil counts in the blood obtained at different stages of the estrous cycle. We found a significant decrease in the percentage of neutrophils counted at d 2 compared to d 1. Even if only a small number of blood samples were analyzed, it appears that this decrease is caused by the simultaneous increase of plasma P4. Changes in circulating populations of neutrophils in response to levels of steroid hormones correspond similarly to changes in activity of WBC counts during the follicular phase as determined previously (Wulster-Radcliffe et al., 2003). It was shown that the numbers of neutrophils per 100 WBC in gilts inoculated with PBS on d 0 decreased significantly over a 72-h sampling period and it was also suggested that this decrease in circulating neutrophils may be attributed to movement of neutrophils out of circulation and into the uterus (Wulster-Radcliffe et al., 2003).

Therefore, our results suggest that ovarian estrogens might be responsible for the initial recruitment of neutrophil granulocytes from the blood but obviously not for the final accumulation within the endometrium when P4 arises. Consequently, local immune-regulatory factors may exist and regulate the cyclic emigration of neutrophils from the blood into the uterine sub- and intraepithelial zones. Different indirect and direct signaling pathways are thereby conceivable. First of all, porcine uterine secretions may contain specific proteins acting on luminal epithelial cells or intraepithelial lymphocytes which in turn might send out cytokines to modulate the sub- and intraepithelial accumulation of neutrophils. For example, transforming growth factors were identified as specific immunologically active proteins in porcine uterine flushings (Gupta et al., 1998). However, the observations of Taylor et al. (2009a) studying the influence of different inseminate components on porcine leucocyte migration indicate that increased uterine secretions by itself could have a stimulatory effect on luminal epithelial cells. By *in vitro* transmigration studies these authors have shown that the influx of PMN after insemination is most probably not on account of direct chemoattractive properties of inseminate components but more likely due to some unspecified irritation of the epithelial cells or resident lymphocyte population (Taylor et al., 2009a).

Second of all, estrogen-primed endometrial cells produce inflammatory cytokines of which IL-8 and GM-CSF are considered as classic neutrophil-recruiting mediators. In this study, G-CSF immunostaining was not detected in endometrial cells of cyclic pigs. Our results agreed with the findings that GM-CSF mRNA is found to be neither expressed (Chabot et al., 2004) nor up-regulated (Taylor et al., 2009b) in the endometrium of cyclic and pre- and postovulatory pigs, respectively. In human and pig endometrium, evidence was given that IL-8 expression seemed to be involved in the regulation of neutrophil recruitment (Arici et al., 1998; Taylor et al., 2009b). However in pigs, a correlation with IL-8 expression was only found in the presence of semen extender but not in untreated cycling pigs (Taylor et al., 2009b). Furthermore, endometrial cells actually producing IL-8 protein were yet not detected in the pig. In human endometrium, IL-8 is mainly localized in glandular cells, suggesting that it may have other functions besides the recruitment of leukocytes (Arici et al., 1998). Collectively, there are yet no comprehensive data showing an effective involvement of GM-CSF or IL-8 in recruitment of neutrophils into the endometrium of the pig during the estrous cycle.

A third signal way for neutrophil recruitment may arise from the cellular growth and differentiation events of the endometrium during the estrous cycle. Under those dynamic tissue remodeling processes, endometrial cells appear to generate stress and that leads to activation of stress-response proteins. In female reproductive organs, small HSPs such as HSP 27 are believed to confer cytoprotective functions under physiological stress (Devaja et al., 1997). In the present study we localized HSP 27 in porcine LE and GE throughout the estrous cycle. Staining intensity of HSP 27 in epithelia varied and appeared to be dependent on the stage of the estrous cycle indicating its putative role in the regulation of cell growth and differentiation. For example, during estrus, HSP 27 immunostaining was specifically strong in LE and faint or absent in GE. This finding correlates with uterine proliferation during estrus which is active in the LE and superficial GE but not in the deep GE (Bazer et al., 2001). In conjunction with neutrophil migration there may be an additional role assigned to this protein. Previously it was shown in experimental models of myocardial ischemia that HSP 27 treatment may promote PMN migration by yet not described mechanism (Légaré et al., 2007). Furthermore, some heat shock proteins are able to bind to and activate PMN which in turn can release large amounts of antimicrobial mediators (Radsak et al., 2003; Osterloh et al., 2009). Our immunohistochemical data show that HSP 27 is strongly expressed in LE during estrus when the infiltration of neutrophils in subepithelial zones of uterine LE increases. HSP 27 immunoreaction in LE is downregulated at d 2 of the estrous cycle after the maximum number of LF-positive neutrophil granulocytes was accumulated, probably due to prevent further accumulation. Taken together, we suggest that in the pig HSP 27 may serve as a chemoattractant for neutrophils during the follicular phase of the estrous cycle and HSP 27-activated neutrophils might release lactoferrin against invading pathogens. Nevertheless, neutrophil migration and incubation assays are required to determine whether HSP 27 serves as a chemoattractant and/or lactoferrin is released by neutrophils upon HSP 27 activation.

5. Conclusion

In conclusion, we detected and quantified neutrophil granulocytes in porcine endometrium during different stages of the estrous cycle using LF as a specific neutrophil marker. Maximum infiltration of neutrophils in intra- and subepithelial zones of uterine luminal epithelium was present at d 2 of the estrous cycle. Our immunohistochemical data correlated with neutrophil counts in the blood being significantly high at d 1 compared to d 2 of the estrous cycle. These findings may reflect the possibility of translocation of neutrophils from the blood into the endometrium up to d 2 of the estrous cycle. Additionally, this paper gives a detailed description of immunolocalization of HSP 27 in the porcine uterus during the estrous cycle. Several roles have been attributed to HSP 27, e.g. as a molecular chaperone (Lanneau et al., 2008), in cell growth and differentiation events (Devaja et al., 1997), and in inflammation and neutrophil infiltration (Légaré et al., 2007). At present, however, there is yet no direct evidence assigned to this protein that elucidates its specific function during neutrophil recruitment.

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Immunohistochemical study on differential distribution of progesterone receptor A and progesterone receptor B within the porcine ovary

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ABSTRACT

Progesterone plays a central role in the regulation of ovarian functions. Progesterone receptor (PGR) is expressed as two isoforms, PGRA and PGRB, which have been shown to have different functional activities. This immunohistochemical study describes the diverse localization of PGRA and PGRB in the porcine ovarian components: small and large antral follicles, early, midluteal and regressing corpora lutea. Ovaries were obtained from 10 cycling sows. PGRs were visualized by immunohistochemistry on paraffin sections using monoclonal antibodies against PGRA (clone 16) or PGRB (clone SAN 27). Moreover, to confirm the specificity of antibodies, Western blot analysis was performed. Nuclear staining for PGRs was observed in cells of granulosa and theca layer, corpora lutea and surface epithelium. Our results revealed that PGRA was the predominant PGR isoform in the pig ovary. In small antral follicles its expression was restricted to theca interna cells, but in large preovulatory follicles, both granulosa and theca layers stained for PGRA. In contrast, immunoreaction for PGRB isoform was limited to granulosa cells of large antral porcine follicles, while their theca layers were devoid of PGRB. The number of both PGRA and PGRB stained cells within the corpora lutea declined significantly during the luteal phase, which might be explained by the decreasing level of locally produced progesterone. The differential distribution and variations in the scores for PGRA and PGRB in various pig ovarian cell types during the cycle may reflect diverse functions for PGRA and PGRB as well as suggest a cell-specific influence of progesterone.

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1. Introduction

The ovary is one of the major target tissues for paracrine/endocrine actions of progesterone (Graham and Clarke, 1997). The diverse biological effects of progesterone on the ovary are mediated by interactions of this hormone with progesterone receptors (PGRs), which are ligand-activated transcription factors and belong to a superfamily of nuclear receptors (Leonhardt and Edwards, 2002). PGR is expressed as two isoforms termed PGRA and PGRB (Leonhardt and Edwards, 2002), that arise from a single gene, each under the control of a separate promoter (Conneely et al., 1989; Kraus and Katzenellenbogen, 1993). In humans PGRA (94 kDa) differs from PGRB (120 kDa) by lacking 164 amino acids at the N-terminus (Kastner et al., 1990). This region has been shown to encode a transactivation function that is specific to PGRB protein (Wen et al., 1994) and plays a crucial role in specifying target genes activated by PGRB, but not PGRA (Horwitz, 1992). Although these isoforms have similar steroid hormone and DNA binding activities, they posses distinct transcriptional activities and co-regulator interactions (Giangrande et al.,

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2000). *In vitro* experiments have shown that the transcriptional activities of the two PGR isoforms vary depending on the cell type. Generally, PGRB acts as a strong activator of transcription of PGR-dependent genes in a variety of cell types in which PGRA is inactive. Moreover, when PGRA and PGRB proteins are coexpressed in cultured cells, the PGRA can impede the PGRB signaling (Giangrande and McDonnell, 1999). Definitive proof that differences of transcriptional function between these two isoforms may result in various biological effects has been provided by the PGRKO mice. Indeed, PGRA null mice, which express PGRB only, demonstrated decreased ovulation rate (Mulac-Jericevic et al., 2000), while those that lacked only the B isoform were not affected (Mulac-Jericevic et al., 2001). Ablation of both isoforms inhibited ovulation (Lydon et al., 1995).

The presence of both isoforms of PGRs has been demonstrated in the ovary of numerous species, e.g. in human (Teilmann et al., 2006), mouse (Gava et al., 2004), cow (D'Haeseleer et al., 2007), and chicken (Gonzalez-Moran and Camacho-Arroyo, 2001). It appears that differential morphological distribution of PGR isoforms may modify the cellular response to progesterone. Although the distribution of PGR within the pig ovary has already been examined in our previous research (Słomczyńska et al., 2000), there is no information as to the specific cellular localization of PGRA and PGRB protein isoforms. Therefore, the aim of the present study was to determine the locations of both PGRs in the porcine follicle and corpus luteum by immunohistochemistry and to confirm the specificity of antibodies used by the Western blot technique.

2. Materials and methods

2.1. Animals and tissue collection

Porcine ovaries were obtained from cycling sows ($n = 10$) at a local slaughterhouse. The follicles were classified as small antral (<5 mm in diameter) and large antral (6–10 mm in diameter). The corpora lutea were collected on days 1–3, 8–10 and 14–16 after ovulation for early stages of development (CL1), midluteal (CL2) and regressing (CL3), respectively. Soon after slaughter, the ovaries were collected and the follicles (4–5 per ovary) and corpora lutea (3–4 per ovary) were excised. The tissues were immediately frozen in liquid nitrogen for further protein isolation or fixed in 10% formalin for immunohistochemical localization of PGRA and PGRB. After fixation, the follicles and corpora lutea were dehydrated in ethanol series, cleared in xylene and processed through Paraplast (Monoject Scientific Division of Scherwood Medical, Saint Louis, MO, USA). Sections 6 μm thick were mounted onto slides covered with APES (3-aminopropyltriethoxysaline).

2.2. Immunohistochemical procedure

The procedures used for immunohistochemistry were similar to those described previously by Słomczyńska and Tabarowski (2001). Dewaxed, hydrated tissue sections were boiled in 0.01 M citrate buffer (pH 6.0) in the microwave oven (750 W, 3 × 4 min) to retrieve antigenicity. After cooling the slides were immersed in 0.3% H_2O_2 in TBS (Tris-buffered saline, pH 7.4) for 30 min to quench endogenous peroxidase activity. To decrease non-specific binding, sections were blocked for 30 min with 5% normal horse serum (for PGRA and PGRB). Then tissues were incubated in a moist chamber overnight at 4 °C with mouse monoclonal antibodies against PGRA clone 16 (1:100; Novocastra Lab, Newcastle, UK) or against PGRB clone SAN 27 (1:100; Novocastra Lab, Newcastle, UK) followed by biotynylated horse anti-mouse IgG (1:300, 1.5 h at RT; Vector Lab., Burlingame CA, USA) and streptavidin-horseradish peroxidase complex (1:100, 40 min at RT in darkness; StreptABComplex-HRP, Dako/AS, Denmark). Peroxidase activity was visualized by incubating with 0.01% H_2O_2, 0.05% diaminobenzidine and 0.07% imidazole in TBS buffer to reveal the brown immunoreactive cells. Finally, the slides were counterstained with Mayer's hematoxylin, dehydrated and mounted in DPX (Fluka Chemie GmbH, Buchs). Negative controls were performed by substituting the primary antibody with a non-immune mouse serum.

2.3. Quantitative evaluation of the staining intensity and statistical analysis

The stained sections were photographed using the Nikon Eclipse E200 microscope attached to the Coolpix 5400 digital camera (Nikon, Japan) with corresponding software. To estimate the intensity of immunoreaction quantitatively, the digital images derived from at least six different sections from each animal examined were analyzed using a public domain ImageJ software. The source images were converted to 8-bit grayscale images and the intensity of staining for PGRA and PGRB was measured separately using the point selection tool. Moreover, the background intensity was also analyzed. Results from each sample were saved as an individual mean and interpolated to the following formula: $\mathrm{ROD} = \mathrm{OD}_{\mathrm{specimen}}/\mathrm{OD}_{\mathrm{background}} = \log(\mathrm{GL}_{\mathrm{blank}}/\mathrm{GL}_{\mathrm{specimen}})/\log(\mathrm{GL}_{\mathrm{blank}}/\mathrm{GL}_{\mathrm{background}})$; where GL—grey level for the stained area (specimen) and the unstained area (background) and blank as a grey level measured after removing the slide from the light path. The intensity of immunohistochemical reaction was expressed as relative optical density (ROD). Statistical analysis was performed using Statistica 5.1 program (StatSoft Inc., USA). All data were examined by one-way analysis of variance (ANOVA). Differences between groups were assayed using the Tukey test. Statistical significances were set at $p < 0.05$.

2.4. Western blot procedure

Small antral follicles, large antral follicles and corpora lutea (CL1, CL2, CL3) excised from porcine ovaries were homogenized on ice with a cold Tris/EDTA buffer (pH 7.5), sonificated and centrifuged at 10,000 × *g* for 20 min at 4 °C as previously described (Knapczyk et al., 2008). Briefly, samples were run on discontinuous polyacrylamide gels (5% stacking and 10% separating gel) under denaturing conditions. After electrophoresis, the proteins in the gels were transferred to nitrocellulose membranes at 250 mA. They were incubated in the presence of monoclonal anti-PGRA or anti-PGRB antibodies (see immunohistochemical procedure) at dilutions of 1:20,000 or 1:1000, respectively for 1.5 h at RT. After washing three times for 10 min in TBST, the membranes were incubated with a horse anti-mouse IgG for 1 h at a dilution of 1:1000. Then they were washed twice for 5 min in TBST and once for 5 min in TBS. Bands were visualized by incubation in TBS containing DAB (0.5 mg/ml) and 0.3% H_2O_2.

3. Results

3.1. Western blot analysis

Western blot analysis was conducted to confirm the specificity of antibodies used. The positive bands of appropriate sizes for both of the studied receptors (PGRA and PGRB) were revealed. The PGRA antibody detected a single band at 94 kDa (Fig. 1A), however in CLs homogenates a faint band around 120 kDa was also observed (isoform B). PGRB was detected as a 120 kDa single band in each sample examined (Fig. 1B).

3.2. Immunolocalization of PGRA and PGRB

The sections of porcine follicles and corpora lutea showed a positive reaction for PGRs proteins predominantly in cell nuclei. However, the pattern of PGRA and PGRB distribution differed significantly in the investigated ovarian components. In small antral follicles, immunohistochemical staining for PGRA was restricted to theca interna cells (Fig. 2A), whereas their granulosa cells revealed lack of immunoreaction. In large preovulatory follicles, both granulosa and theca layers stained for PGRA (Fig. 2C). The intensity of reaction exhibited by granulosa cells of large antral follicles was very high, however there were no differences in the intensity of staining for PGRA within theca cells of both classes of follicles.

The present study demonstrated also nuclear staining for PGRA in the large luteal cells of all investigated corpora lutea (Fig. 3A, C, E). Moreover, the number of stained cells within the corpora lutea declined significantly during the luteal phase. The most intensive reaction for PGRA was observed in early corpus luteum CL1 (Fig. 3A), whereas in midluteal CL2 (Fig. 3C) and regressing CL3 (Fig. 3E) only some cells were immunostained with weaker intensity.

Additionally, the pig ovarian surface epithelium indicated very strong immunostaining for PGRA (Fig. 2E).

In contrast to PGRA expression, staining for PGRB protein was limited to granulosa layer of large antral follicles (Fig. 2D), where cells showed weak intensity of immunohistochemical reaction. Theca layer of pig follicles of both sizes was devoid of the immunoreactivity for PGRB. Moreover only few CL1 (Fig. 3B) and CL2 (Fig. 3D) cells showed relatively weak nuclear staining, while CL3 indicated cytoplasmatic localization of PGRB (Fig. 3F). In the ovarian surface epithelium (Fig. 2F) immunohistochemical reaction for PGRB was present in some cells.

Negative control sections incubated with mouse serum showed nonspecific staining neither for PGRA (Fig. 2A, insert) nor for PGRB (Fig. 3A, insert). Comparison of the intensity of PGRA and PGRB nuclear immunostaining in pig ovarian structures is summarized in Table 1 and shown in Fig. 4.

4. Discussion

In our previous study of PGR expression in the porcine ovary (Słomczyńska et al., 2000) the antibody that recognized both PGR isoforms was applied. However, Western blotting analysis showed that two bands corresponding to the PGR isoforms A and B were present. Using antibodies specifically recognizing PGRA and PGRB, we were able to demonstrate both proteins to be differentially expressed in the porcine ovary during the estrous cycle.

Both PGRA and PGRB are capable of binding progesterone and after that can form dimers (homo- or heterodimers). The ratio of PGRA and PGRB proteins within a target cell, and contribution of different transcription factors to their transactivation, are responsible for the cellular response to progesterone (Conneely et al., 2003). The presence of PGRs in the granulosa and theca layer of preovulatory follicles identifies them as targets for progesterone action. We observed PGRA expression only in theca cells of small antral follicles, while both PGRA and PGRB proteins were detected in granulosa and theca layers of preovulatory follicles. PGRA protein level was much greater than that of PGRB. These findings are in agreement with an experiment with LH-stimulated rat granulosa cells *in vitro*, showing a 3-fold increase in PGRA over PGRB (Natraj and Richards, 1993). Transfection studies in rat granulosa cells revealed the presence of a promoter region which is LH responsive (Sriraman et al., 2003). It explains the observation that PGRA was detected in the granulosa cells of mice preovulatory follicles but not at any other stage of follicle development (Gava et al., 2004). In contrast, PGRB was detected in the granulosa and theca cells at all stages of follicle development during the estrous cycle. In LH-stimulated porcine small antral follicles in culture, the expression of PGR was observed (Słomczyńska et al., 2000),

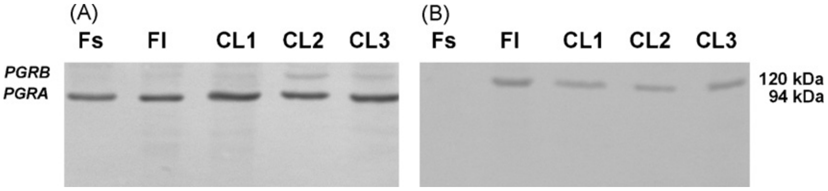

Fig. 1. Western blot analysis of PGRA and PGRB protein extracts of the porcine ovaries. The specific antibody against PGRA (A) revealed a 94 kDa protein band (for A isoform). However, in some samples also a 120 kDa band (for B isoform) was observed. The specific anti-PGRB antibody showed as only one 120 kDa protein band. (B) Fs—small antral follicle; Fl—large antral follicle; CL1—early corpus luteum; CL2—midluteal corpus luteum; CL3—regressing corpus luteum.

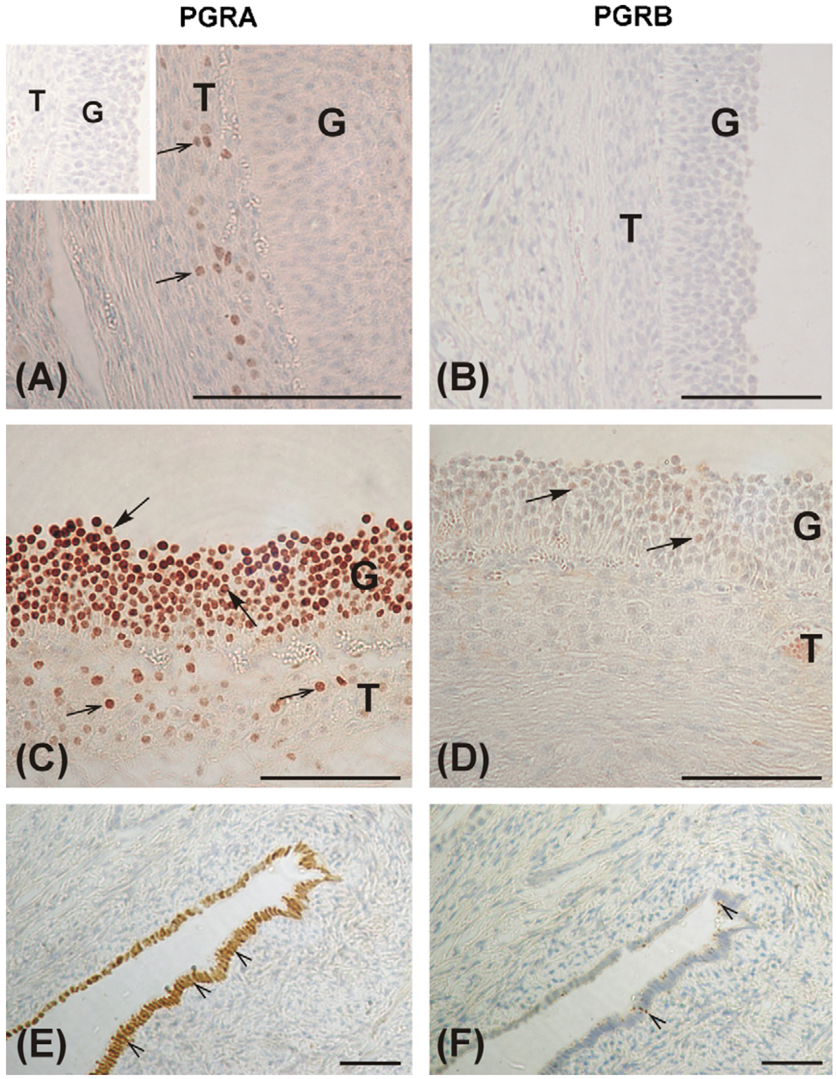

Fig. 2. Localization of PGRA and PGRB protein in the paraplast sections of pig ovarian follicles of different sizes: small antral (A, B), large antral (C, D) and in the surface epithelium (E, F). In the small antral follicles immunoreaction for PGRA (A) was observed exclusively in the nuclei of theca cells (open arrows), while PGRB was absent (B). In the large antral follicles intensive staining for PGRA (C) was localized in the nuclei of both granulosa (black arrows) and theca cells (open arrows), whereas immunoreactivity for PGRB (D) was weak and present exclusively in granulosa cells (black arrows). Moreover PGRA (E) and PGRB (F) indicated nuclear immunolocalization in the porcine ovarian surface epithelium (open arrowheads). Negative control (A, insert). Bar = 100 μm. G—granulosa cells; T—theca interna cells.

which confirmed the role of this gonadotropin in the receptor signaling pathway. Results obtained from PGRAKO mice clearly indicated that PGRA expressed in granulosa cells of preovulatory follicles was critical for ovulation (Mulac-Jericevic et al., 2000). PGRB, which was also present in the same follicles, was not obligatory for ovulation (Mulac-Jericevic et al., 2003). Therefore, the observed changes in the ratio of receptor expression may be responsible for the cell specific response to steroid hormones and the efficient ovulation (Duffy et al., 1997; Graham and Clarke, 2002). Additionally, Shimada et al. (2004) revealed that the PGR-dependent pathway is essential for normal *in vitro*

Table 1
The intensity of nuclear immunohistochemical staining for PRA and PRB in the sections of the porcine follicles and corpora lutea expressed as relative optical density (ROD) of diaminobenzidine reaction products. Values are means ± SD. Different letters superscripts indicate differences at $p < 0.05$ (ANOVA, Tukey test). Gc—granulosa cells; Tc—theca interna cells; CL1—early corpus luteum; CL2—midluteal corpus luteum; CL3—regressing corpus luteum.

	Small antral follicles		Large antral follicles		Epithelium	CL1	CL2	CL3
	Gc	Tc	Gc	Tc				
PRA	–	1.75 ± 0.543^a	3.71 ± 0.532^b	1.78 ± 0.324^a	3.27 ± 0.432^b	5.12 ± 0.432^a	2.98 ± 0.652^b	0.77 ± 0.532^c
PRB	–	–	1.75 ± 0.432^a	–	1.31 ± 0.321^a	0.22 ± 0.12^d	2.14 ± 0.432^b	–

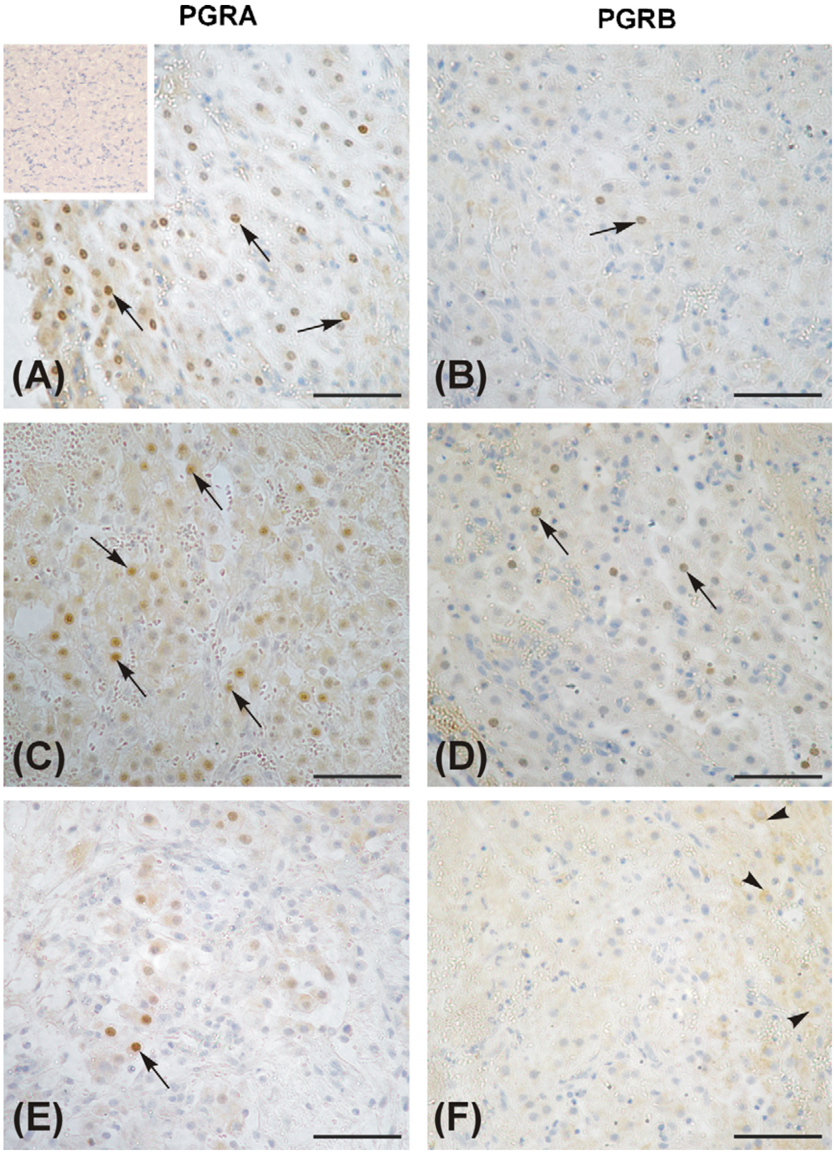

Fig. 3. Localization of PGRA and PGRB protein in the paraplast sections of the pig corpora lutea at different stages of maturation: early CL (A, B), midluteal CL (C, D) and regressing CL (E, F). PGRs proteins were found in the nuclei (black arrows) or cytoplasm (arrowheads) of large luteal cells. Both PGRA and PGRB was localized in all types of porcine corpora lutea. Negative control (A, insert). Bar = 100 μm. CL—corpus luteum.

maturation of porcine oocytes. Their results suggested that the expression of PGR isoforms in cumulus cells have an impact on their proliferation and differentiation, which is crucial for meiotic resumption.

It is worthy of note that there are differences of PGR expression among the species during follicular development. In the rodent and primate, follicles developing beyond the primary stage expressed PGR in granulosa cells after the LH surge (Hild-Petito et al., 1988; Shao et al., 2003). In pig, the level of PGRs in granulosa cells increased after FSH stimulation (Słomczyńska et al., 2000). Yoshimura et al. (1995) showed in chicken ovary that both PGRA and PGRB were expressed in small antral follicles but only PGRA was observed in large antral follicles. Therefore, our present study demonstrate the presence of PGRA in both the theca and granulosa cells, and PGRB in theca cells throughout folliculogenesis. Their expression seem to be dependent on the stage of the estrous cycle.

Progesterone acting through its receptors regulates development and functions of the corpus luteum (CL) (Sasano and Suzuki, 1997). In the current study we observed that the number of cells stained for PGRA and PGRB within the subsequent generations of CLs varied during the luteal phase, which might be explained by the level of locally produced progesterone. These findings are in accordance with results of research performed on monkeys (Hild-Petito et al., 1988), but are in contrast with previous reports concerning rabbits (Korte and Isola, 1988),

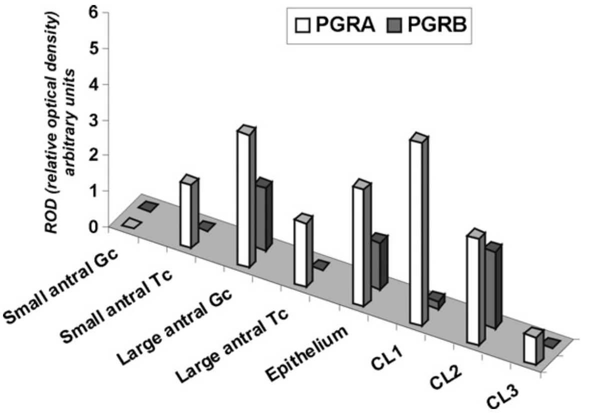

Fig. 4. Expression of PGRA and PGRB in granulosa and theca cells of small and large antral follicles, and corpora lutea during the pig estrous cycle. The expression of PGRA and PGRB is represented as a relative optical density (ROD). Gc—granulosa cells; Tc—theca interna cells; CL1—early corpus luteum; CL2—midluteal corpus luteum; CL3—regressing corpus luteum.

dogs (Vermeirsch et al., 2001) and cows (Van den Broeck et al., 2002). It was noticed that PGR expression in CL was negatively correlated with progesterone concentration due to negative effects of this hormone on the expression of its own receptors. Moreover, data from studies on rodents showed that the PGR mRNA or protein have not been observed in the rat CLs neither at the time of elevated hormone level (Natraj and Richards, 1993) nor during pregnancy (Park-Sarge et al., 1995; Telleria et al., 1999). However, both isoforms were found after ovulation in the developing mouse CL on the morning of estrus. Interestingly, in CL on the morning of metestrus and in CL from the previous cycle only PGRB was present (Gava et al., 2004). Therefore, diverse hormone levels and CL functions result in species specific differences in PGRs expression. In contrast to all other ovarian cells, we demonstrated that the luteal cells of porcine regressing CLs showed not only nuclear but also cytoplasmatic PGRB immunostaining. According to D'Haeseleer et al. (2007) this type of staining may reflect the cycle-dependent localization of PGR. It is not clear whether the receptor is unbound and inactive in cells producing low amount of progesterone or it is ready to be ubiquitinated and released from the cell.

In the present study the very strong immunostaining of PGRA was identified in the porcine ovarian surface epithelium (OSE), while PGRB exhibited very low expression. This corresponds with studies on ovine (Murdoch, 1998), bovine (D'Haeseleer et al., 2007) and primates (Hild-Petito et al., 1988; Chandrasekher et al., 1994). It has been suggested that OSE cells are enzymatically involved in the ovulation under the influence of progesterone and its receptors. On the other hand, PGRs were not detected in the mice OSE cells (Gava et al., 2004). These data, including our own study, indicate that the role of PGRs in OSE should be investigated more thoroughly in each species. Additionally, there is increasing evidence that steroid hormones, including progesterone, regulate growth of ovarian surface epithelium as well as that they are involved in the development of ovarian tumors (Leung and Choi, 2007). Expression patterns of PGRA and PGRB may be a favorable marker in ovarian cancer, especially in light of studies indicating that down-regulation of PGRA is associated with the development of OSE derived carcinoma (Akhaira et al., 2002).

5. Conclusions

In conclusion, our data clearly demonstrate the differential expression of PGRA and PGRB in the porcine ovary during the estrous cycle, which may reflect different functions of PGR isoforms as well as suggesting cell-specific influences of progesterone. Secondly, within the porcine ovary predominantly PGRA was observed both in follicular and luteal cells. This new information can be useful in understanding the mechanism of progesterone action in the porcine ovary, however it requires molecular study.

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Royal jelly counteracts bucks' "summer infertility"

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ABSTRACT

Exposure of male rabbits to heat stress during summer adversely affects their fertility leading to major production losses. A total number of 24 male rabbits were randomly divided into four experimental groups exposed to temperatures ranging from a high of 32 °C to a low of 23 °C. Animals of the 2nd, 3rd and 4th group were individually orally given 200, 400, or 800 mg royal jelly (RJ)/kg body weight once a week to evaluate the ability of RJ feeding to counteract "summer infertility" in bucks and enhance their physiological status. Royal jelly treatments significantly boosted testosterone level to 133, 143 and 124% of basal, increased ejaculated volume by 36, 31 and 18%, increased seminal plasma fructose to 122, 124, and 111%, improved sperm motility by 15, 18 and 5%, increase sperm total output by 65, 63 and 35%, reduced abnormal sperm by 24, 24 and 15% and dead sperm by 27, 25 and 17% compared to the heat stressed control animals. Serum total protein, albumin and globulin increased while serum total lipids, cholesterol and triglycerides decreased with RJ treatments. Creatinine was reduced by 5, 13 and 8% and uric acid by 4, 7 and 4%, respectively for the three doses of RJ compared to control. Alkaline phosphatase has significantly increased to reach 114, 118, and 108% of heat stressed level with the three doses of RJ, indicating the occurrence of active bone deposition. Glucose level increased significantly to reach 105, 112, and 116% of heat stressed control and both calcium and phosphorus increased significantly with RJ treatments. It was concluded that royal jelly administration to heat stressed male rabbits can counteract their "summer infertility" and improve their physiological status.

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1. Introduction

Rabbits are very susceptible to heat stress, since they have few functional sweat glands and have difficulty in eliminating excess body heat. Subjecting male rabbits to heat stress has a significant adverse effect on blood plasma testosterone, ejaculate volume, sperm motility, viability and concentration and semen fructose concentration. High temperature also increase rabbit blood and semen cholesterol, phospholipids, total lipid concentrations and transaminases activities and decreased blood total protein and albumin (Tharwat et al., 1994; El-Masry et al., 1994; Marai et al., 2005, 2008). In these studies there was also a decline in the mean conception rate and litter size at birth compared with the winter season. Physiological changes reported under heat stress conditions include decreases in plasma glucose, thyroid hormone T3, sodium, potassium, calcium, magnesium and phosphorus and significant increases in plasma urea-N and creatinine.

On the other hand, royal jelly (RJ) is a honey bee secretion that is used in the nutrition of the larvae. It is secreted from the hypopharyngeal glands in the heads of young workers and used (among other substances) to feed the larvae in the colony. It is collected as a human dietary supplement, claiming various health benefits because of the B-complex vitamin content. The overall composition of royal jelly is 67% water, 12.5% crude protein (including small amounts of many different amino acids), and 11% simple sugars (monosaccharide), also including 5% fatty acids. It also contains many trace minerals, some

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enzymes, antibacterial and antibiotic components, and trace amounts of vitamin C. Vitamins A, D, E and K are completely absent from royal jelly (Graham, 1992). RJ has been reported to contain testosterone and to have steroid hormone-type activities (Hidaka et al., 2006). Moreover, El-Banby et al. (1987) reported that when royal jelly was administered as a 10% solution either intraperitoneally or through a stomach tube to male rats it stimulated the production of luteinizing hormone, testosterone and progesterone. Results also showed that the administration of royal jelly orally was more beneficial than by injection. It has also been shown to inhibit lipid peroxidation both in vitro and in vivo (Hang et al., 2008), and has antioxidant properties that may play an important role on its effects on aging process and stress reactions in rats (Ikeda et al., 1996)

The present study was conducted to evaluate the ability of royal jelly feeding to counteract “summer infertility” in male rabbits and enhance their physiological status.

2. Materials and methods

The present study was carried out during the summer season (July and August) at the Poultry Research Center, Poultry Production Department, Faculty of Agriculture, Alexandria University, Egypt.

A total number of 24, male rabbits aged 6 months were randomly divided into four experimental groups (6 rabbits each). Rabbits were housed in galvanized wire cages provided with feeders and an automatic watering system, in a well ventilated building and were kept under the same managerial, hygienic and environmental conditions. The temperature ranged from a high of 33 °C to a low of 23 °C and relative humidity ranged from a high of 65% to a low of 53.5%. The basal diet was formulated to cover the nutrient requirements of mature rabbits recommended by (NRC, 1977). Animals of the 2nd, 3rd and 4th group were individually orally given 200, 400, or 800 mg royal jelly (RJ) mixed with distilled water/kg body weight (bwt) once a week, respectively. The first group served as control and was treated in a like manner except that animals were given distilled water only. The experiment lasted 6 weeks.

2.1. Data collected

Blood samples were taken from each rabbit every 2 weeks for biochemical analysis. Hemoglobin (Hb) concentration was measured according to Drew et al. (2004). Serum total protein, albumin, triglyceride and total lipids were measured by means of spectrophotometer according to Armstrong and Carr (1964), Doumas et al. (1977) and Fringes et al. (1972). Globulin values were obtained by subtracting albumin values from the corresponding values of total protein. Serum total cholesterol was determined according to Bogin and Keller (1987). The transaminase enzymes activities of aspartate amino transferees (AST) and alanine amino transferees (ALT) as (U/dl) and alkaline phosphatase were determined by calorimetric method of Reitman and Frankel (1957) and Bauer (1982). Plasma amylase activity and creatinine were assayed according to Caraway (1959, 1963).

Serum testosterone was determined by enzyme immunoassay using commercial kits purchased from Biosource. Plasma glucose concentrations were measured by the Trinder (1969) method. Uric acid was determined colorimetrically according to Arliss and Entristle (1981). Serum calcium and inorganic phosphorus concentrations were determined according to Tietz (1986).

Semen specimens were collected biweekly using an artificial vagina. Ejaculated volume was measured to the nearest 0.01 ml. A weak eosin–formalin (10% formalin) solution was used for evaluation of sperm concentration by the improved Newbuer hemocytometer slide as described by Smith and Mayer (1955). Sperm motility was estimated at 400× magnification (Kamar, 1960). Evaluation of seminal initial fructose was carried out immediately after collection according to Mann (1948). Assessments of dead and abnormal spermatozoa were performed using an eosin–aniline blue staining mixture (Shaffer and Almquist, 1948).

2.2. Statistical analysis

Data were analyzed by analysis of variance using the general linear model procedure (Proc GLM; SAS Institute, 1996). For the overall means, data was classified according to 4 treatments and the mean of each treatment was used. Interaction between time and treatment was confirmed by repeated measures analysis and only significant interactions ($p < 0.001$) were plotted. Differences among means were determined using Duncan test (Duncan, 1955).

3. Results

3.1. Reproductive status

Royal jelly treatments significantly ($p < 0.001$) boosted testosterone level (Table 1) as it reached 133, 143 and 124% compared to the heat stressed control animals, with the three doses of RJ, respectively. Interaction of RJ treatments with weeks (Fig. 1) showed significant effect ($p = 0.0017$) on testosterone level, as it increased in all groups gradually by time. with the highest values observed at the end of the experiment period with the 200 and 400 mg RJ/kg bwt treatments.

Ejaculated volume (Table 1) increased significantly by 36, 31 and 18% compared to the heat stressed control with the three RJ doses, respectively. Seminal plasma fructose (Table 1) level has also increased significantly ($p < 0.001$) with the RJ treatments to reach 122, 124, and 111% of the control level. Sperm motility (Table 1) has significantly ($p < 0.001$) increased due to RJ treatments the enhancement was by 15, 18 and 5% compared to the heat stressed control.

Royal jelly treatments significantly ($p < 0.001$) increased sperm concentration (Table 1) by 17, 19 and 12%, that was also reflected on the sperm total output which increased significantly ($p < 0.001$) and dramatically by 65, 63 and 35% compared to the heat stressed control with the three RJ doses, respectively. All groups showed a significant increase in sperm concentrations and total output overtime with significant interaction (Figs. 2 and 3). The highest val-

Table 1
Mean (±S.E.) of testosterone and some semen characteristics of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

	Testesterone (ng/ml)	Ejaculated volume (ml)	Seminal plasma fructose (μg/ml)	Motility (%)
Control (HS)	2.84 ± 0.06^{D}	0.45 ± 0.01^{C}	676 ± 4.9^{C}	66.25 ± 1.08^{C}
HS + 200 mg RJ	3.77 ± 0.09^{B}	0.61 ± 0.02^{A}	823 ± 8.7^{A}	76.25 ± 0.89^{A}
HS + 400 mg RJ	4.07 ± 0.13^{A}	0.59 ± 0.03^{A}	836 ± 7.7^{A}	77.92 ± 1.44^{A}
HS + 800 mg RJ	3.51 ± 0.08^{C}	0.53 ± 0.03^{B}	751 ± 7.9^{B}	69.58 ± 1.31^{B}
p value	0.0001	0.0001	0.0001	0.0001
	Sperm concentration (10^6 ml^{-1})	Total sperm output (10^6)	Abnormal sperm (%)	Dead sperm (%)
Control (HS)	134 ± 3.12^{C}	59.11 ± 2.96^{C}	13.08 ± 0.26^{A}	11.67 ± 0.22^{A}
HS + 200 mg RJ	157 ± 5.76^{A}	97.26 ± 6.36^{A}	10.00 ± 0.40^{C}	8.50 ± 0.53^{C}
HS + 400 mg RJ	160 ± 6.10^{A}	96.18 ± 7.98^{A}	9.91 ± 0.29^{C}	8.75 ± 0.52^{C}
HS + 800 mg RJ	150 ± 5.59^{B}	79.93 ± 6.05^{B}	11.17 ± 0.44^{B}	9.67 ± 0.39^{B}
p value	0.0001	0.0001	0.0001	0.0001

Different letters (A, B, C) within a column denote significant differences between treatments ($p < 0.05$).

ues were observed at the end of the experimental period with the 200 and 400 mg RJ/kg bwt treatments.

Abnormal and dead sperm concentrations (Table 1) were affected ($p < 0.001$) by royal jelly treatments. Abnormal sperm showed a reduction of 24, 24 and 15% and dead sperm was reduced by 27, 25 and 17% compared to the heat stressed control with the three doses of RJ, respectively. The interaction of RJ treatments with weeks was significant for dead sperm concentrations (Fig. 4), where the lowest values were observed at the end of the experimental period with the 200 and 400 mg RJ/kg bwt treatments.

3.2. *Physiological status*

Serum total protein (Table 2) increased significantly ($p < 0.001$) to reach 114, 116 and 114% of controls' value, similar trend was observed with serum albumin as it reached 110, 112, and 118% ($p = 0.0477$) and serum globulin which reached 120, 122, and 108% of controls' value ($p = 0.0004$) with the three RJ doses, respectively. Interaction of RJ treatments with weeks was not significant.

Royal jelly treatments significantly ($p = 0.0001$) reduced serum total lipids (Table 2) by 5, 6 and 9% compared to the heat stressed control with the three doses, respectively. Similar trend was observed with cholesterol ($p = 0.0033$) as it was reduced by 7, 15 and 14% compared to control with the three doses of RJ, respectively.

Royal jelly treatments caused a reduction in ALT level ($p < 0.01$, Table 2), as ALT was reduced to reach almost 94, 98 and 97% of its heat stress level, with the three royal jelly doses, respectively. Moreover, gamma glutamyl transpeptidase decreased significantly ($p = 0.0001$) with RJ treatments by 8, 12 and 10% compared to the heat stressed control rabbits.

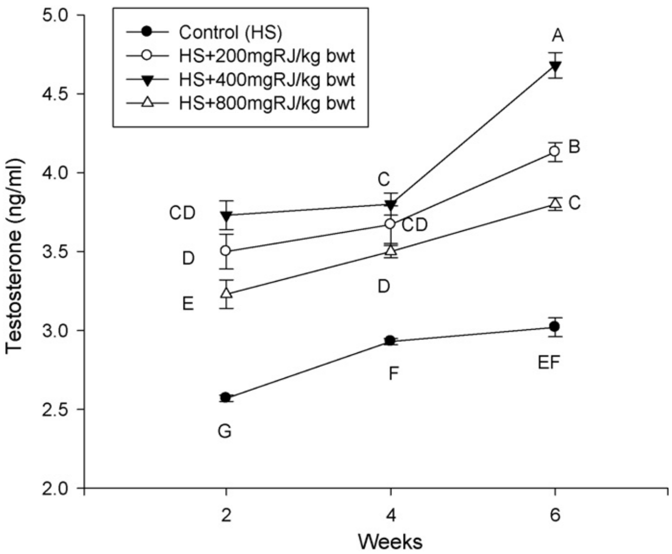

Fig. 1. Mean (±S.E.) of biweekly testosterone concentrations (ng/ml) of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

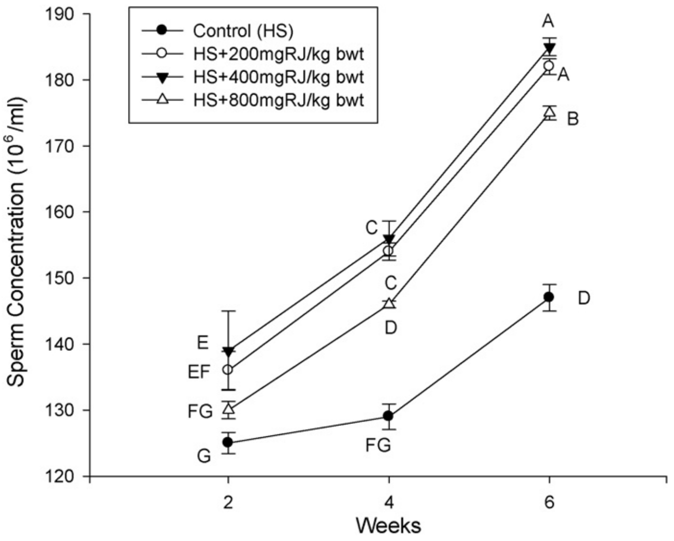

Fig. 2. Mean (±S.E.) of biweekly sperm concentrations (10^6 ml^{-1}) of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

Creatinine and uric acid concentrations (Table 2) were affected ($p = 0.0001$ and 0.1678, respectively) by royal jelly treatments. Creatinine showed a reduction of 5, 13 and 8% and uric acid was reduced by 4, 7 and 4% compared to the heat stressed control with the three doses of RJ, respectively.

Alfa amylase levels (Table 2) increased significantly ($p < 0.001$) in a dose dependent manner to reach 115, 120 and 127% of heat stressed levels with the three doses of RJ, respectively. Glucose level increased significantly ($p = 0.0001$) with RJ treatments to reach 105, 112, and 116% of heat stressed control level, with the three doses of RJ, respectively.

Alkaline phosphatase (ALP) (Table 2) has significantly increased ($p < 0.001$) to reach 114, 118, and 108% of heat stressed level with the three doses of RJ, respectively. Both calcium and phosphorus increased significantly with RJ treatments maintaining a constant ratio ($p < 0.001$). The increase was by 9, 15, and 18% in calcium and was by 19, 26, and 19% in phosphorous with the three RJ doses, respectively.

Red blood cell count has increased significantly ($p < 0.001$) to reach 126, 128, and 112% compared to the heat stressed control animals with the three doses of RJ, respectively (Fig. 5). Royal jelly treatment caused a slight increase in hemoglobin (Fig. 5), which was accompanied

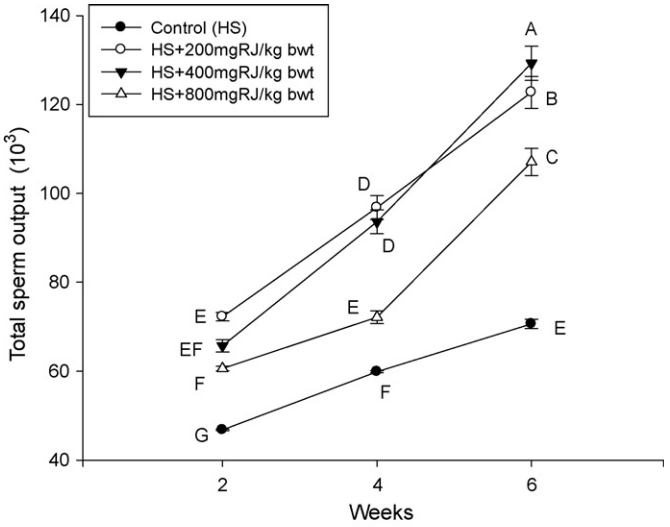

Fig. 3. Mean (±S.E.) of biweekly total sperm output (10^6) of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

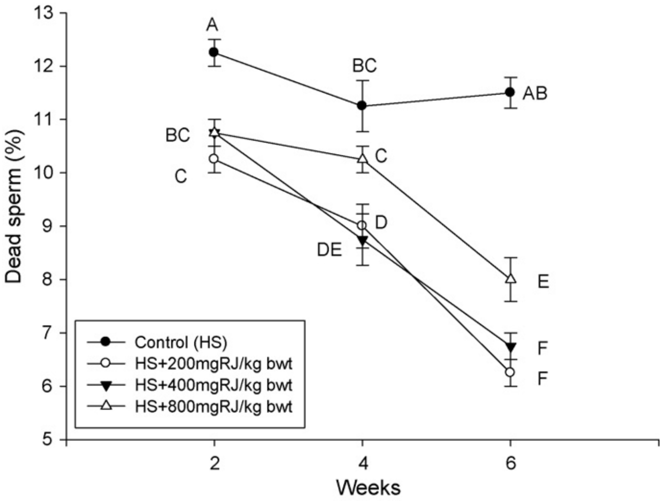

Fig. 4. Mean (±S.E.) of biweekly dead sperm percent (%) of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

with a significant ($p = 0.0127$) increase in packed cell volume (PCV) to reach about 104% of heat stressed rabbits' values with the three doses of RJ.

4. Discussion

4.1. Reproductive status

Results of this study show that the impaired reproductive status of bucks observed under heat stress, can be enhanced by royal jelly administration. Although, RJ effects have never been studied under heat stress before, its effect on testosterone comes in agreement with the findings of Kohguchi et al. (2004) who demonstrated that feeding golden hamsters with diet containing RJ showed higher testosterone than the control group in a dose-dependent manner and indicated that long-term feeding of RJ inhibits the age-associated decline in the testicular function of male hamsters. The increase observed in this study in both ejaculate volume and seminal plasma fructose is in accordance with the effect of testosterone reported by Nishiyama (1955) and Fujihara et al. (1983). Hafez (2000) also concluded that fructose synthesis by the accessory sex glands was dependent on the secretion of testosterone hormone by testes laydig cell. The enhancement observed in sperm motility is consistent with the findings of Karacal and Aral (2008) who reported higher sperm motility when male mice were treated with RJ. The significant increase in sperm concentration and therefore sperm total output can be explained by the findings of (Kohguchi et al., 2004) who demonstrated that golden hamsters treated with RJ showed more intensive spermatogenesis than the con-

Table 2
Mean (±S.E.) of some blood constitutes of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Total lipids (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control (HS)	3.94 ± 0.13^{B}	2.43 ± 0.08^{B}	1.51 ± 0.05^{B}	235 ± 6.51^{A}	67.15 ± 0.59^{A}	147.1 ± 8.51
HS + 200 mg RJ	4.49 ± 0.08^{A}	2.68 ± 0.13^{AB}	1.81 ± 0.07^{A}	224 ± 6.90^{B}	62.73 ± 2.49^{AB}	135.7 ± 6.85
HS + 400 mg RJ	4.57 ± 0.10^{A}	2.73 ± 0.13^{AB}	1.84 ± 0.06^{A}	221 ± 6.42^{B}	57.07 ± 2.08^{B}	137.8 ± 7.63
HS + 800 mg RJ	4.49 ± 0.11^{A}	2.86 ± 0.13^{A}	1.63 ± 0.05^{B}	214 ± 6.09^{C}	57.68 ± 2.96^{B}	137.2 ± 7.62
p value	0.0001	0.0477	0.0004	0.0001	0.0033	0.6357

	AST (IU/l)	ALT (IU/l)	y-GT (IU/l)	Creatinine (mg/dl)	Uric acid (mg/dl)	Alfa amylase (IU/l)
Control (HS)	30.09 ± 0.34	14.03 ± 0.16^{A}	678 ± 22.2^{A}	1.15 ± 0.02^{A}	7.44 ± 0.29	316 ± 3.41^{D}
HS + 200 mg RJ	29.97 ± 0.26	13.18 ± 0.22^{C}	625 ± 21.4^{B}	1.09 ± 0.01^{B}	7.13 ± 0.12	364 ± 3.92^{C}
HS + 400 mg RJ	30.14 ± 0.14	13.86 ± 0.30^{AB}	597 ± 16.0^{D}	1.00 ± 0.03^{C}	6.94 ± 0.07	379 ± 4.95^{B}
HS + 800 mg RJ	30.01 ± 0.19	13.71 ± 0.24^{B}	613 ± 14.5^{C}	1.06 ± 0.01^{B}	7.15 ± 0.11	401 ± 4.61^{A}
p value	0.9107	0.0001	0.0001	0.0001	0.1678	0.0001

	Glucose (mg/dl)	Alkaline phosphatase (IU/L)	Calcium (mg/dl)	Phosphorous (mg/dl)
Control (HS)	57.66 ± 0.50^{D}	60.64 ± 0.99^{D}	8.08 ± 0.12^{C}	3.81 ± 0.09^{C}
HS + 200 mg RJ	60.43 ± 0.69^{C}	69.25 ± 1.75^{B}	8.81 ± 0.10^{B}	4.53 ± 0.11^{B}
HS + 400 mg RJ	64.81 ± 1.07^{B}	71.38 ± 0.77^{A}	9.32 ± 0.33^{A}	4.81 ± 0.14^{A}
HS + 800 mg RJ	66.80 ± 1.34^{A}	65.73 ± 0.73^{C}	9.53 ± 0.31^{A}	4.52 ± 0.17^{B}
p value	0.0001	0.0001	0.0001	0.0001

Different letters (A, B, C) within a column denote significant differences between treatments ($p < 0.05$).

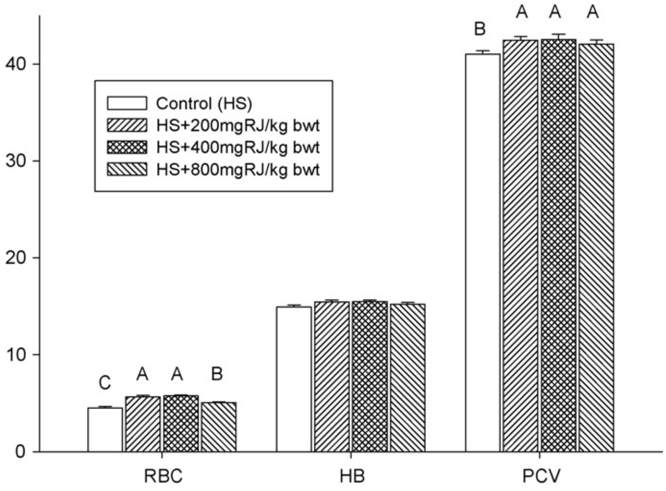

Fig. 5. Mean (±S.E.) of some blood hematology of heat stressed (HS) male rabbits treated with 200, 400 and 800 mg royal jelly (RJ)/kg body weight.

trol group. Similar observations were recorded by Karacal and Aral (2008) who reported higher sperm concentration when male mice were treated with RJ.

The present results show a gradual increase with time in testosterone, sperm concentration and total output of the heat stressed control group suggesting a slight adaptation to the environment but in all cases remained significantly lower than RJ treated groups.

In addition to the previous beneficial effects of RJ on heat stressed males' reproductive status, RJ treatments were capable of significantly reducing abnormal and dead sperm concentrations. That comes in harmony with the findings of Karacal and Aral (2008) who reported lower abnormal sperm concentration when male mice were treated with RJ, where the abnormal spermatozoa rate in the control group was more than 3-fold higher than that in the experimental group.

Improvements in buck reproductive status as a result of RJ treatments can possibly be attributed to different amino acids and/or vitamins (Graham, 1992), as discussed by Nizza et al. (2000). Alvarez et al. (2006a,b) also reported that feeding rabbits rations containing different levels of vitamin C improved spermatic concentration and reduced abnormal sperm count as a result of antioxidant protective effects. RJ was shown to contain testosterone and to have steroid hormone-type activities (Hidaka et al., 2006) may also be the reason for its beneficial effects on rabbits' reproductive status. In the same way ElKelawy et al. (1995) reported that ejaculate volume, sperm motility and sperm concentration were significantly increased when male rabbits were treated with testosterone.

Royal jelly treatments reduced heat stressed rabbits total lipids and cholesterol supporting the findings of XinNan et al. (1995) who found that treating rats with experimentally induced hyperlipaemia with 700 mg RJ/kg reduced serum cholesterol levels. Vittek (1995) also reported a reduction in serum lipids in rats, rabbits and humans treated with royal jelly. Likewise Al-Mufarrej and El-Sarag (1997) reported that treating chickens with 200 mg RJ reduced blood cholesterol.

5. Conclusion

It can be concluded that royal jelly administration to heat stressed male rabbits can really counteract their "summer infertility", by improving their performance as observed with higher testosterone levels, ejaculated volume, seminal fructose, sperm motility and total sperm output, also by decreasing abnormal and dead sperm concentrations. This improvement was also mirrored on rabbits' physiological status, indicating better liver and kidney functions.

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Temperature-dependent regulation of sperm motility of Ijima's copper pheasants (*Syrmaticus soemmerringii ijimae*), one of 'near threatened' species

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ABSTRACT

In order to conserve the copper pheasants, one of the Japanese 'near threatened' species, the knowledge on the sperm characteristics is the inevitable issue. Therefore, temperature-dependent regulation of copper pheasant sperm motility was investigated in comparison with that of domestic fowl spermatozoa. Motility of intact spermatozoa from both species was markedly affected by temperature. During incubation at 30 °C, copper pheasant spermatozoa showed around 60–70% motility, but became almost immotile when the temperature was raised to 40 °C. Then, when the temperature of the sperm suspension was subsequently cooled to 30 °C, the spermatozoa regained their motility. The motility of domestic fowl spermatozoa showed a similar pattern. Temperature also affected the motility of both demembranated copper pheasant and domestic fowl spermatozoa in the same way. The motility of intact copper pheasant and domestic fowl spermatozoa at 30 °C was unaffected following the addition of 2 mM $CaCl_2$, 100 nM calyculin A, an inhibitor of protein phosphatase-type 1 (PP1), or 4 mM diB-cAMP, respectively, compared with those with no effectors. However, the presence of 10 μM ML-7, a selective inhibitor of myosin light chain kinase (MLCK), inhibited motility of spermatozoa from both species. At 40 °C, the presence of $CaCl_2$ or calyculin A restored the motility of spermatozoa from both species, but the addition of diB-cAMP or ML-7 could not prevent the immobilization of spermatozoa. At 30 °C in the presence of ATP, the motility of demembranated copper pheasant spermatozoa was over 60% but was inhibited following the addition of 10 μM ML-7; a similar pattern was found with demembranated domestic fowl sperm motility. The motility of demembranated spermatozoa from both species was inhibited following the addition of 2 mM EGTA to the reactivation medium at 30 °C, but restored by the subsequent addition of 4 mM $CaCl_2$. These results suggest that copper pheasant sperm motility might be regulated by similar mechanisms to that of domestic fowl spermatozoa: i.e., the balance of Ca^{2+}/MLCK or an MLCK-like protein-dependent phosphorylation and PP1-dependent dephosphorylation. The similarity in physiological regulation of spermatozoa from both species shows that extensive technology developed for artificial breeding of the domestic fowl might be applicable to captive breeding of copper pheasants.

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1. Introduction

The copper pheasants (*Syrmaticus soemmerringii*) are native to Japan and inhabit coniferous forest and adjoining

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mixed forest with dense undergrowth and grassy hillsides in mountainous regions at elevations below around 1300 m. Their diet consists mainly of acorns and mast of such forest trees, which are gathered from the forest floor. They also eat many kinds of insects, earthworms and crabs (Johnsgard, 1999). Due to ongoing habitat loss, limited range and overhunting in some areas, the copper pheasant is evaluated as 'Near Threatened' on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (IUCN Red List, 2008).

The Ijima (*S. soemmerringii ijimae*) is distributed in southern Kyushu and is one of five subspecies. The male is easily recognized by the large white patch on the lower back and rump. He has a beautiful long tail (around 70–80 cm), about twice the length of his body. As its name suggests, the colour of the male is a coppery reddish brown which with the tiniest ray of light shines more like gold. The female is a very pretty bird, although she lacks the iridescence and long tail of her male counterpart. She is heavily flecked with many shades of brown and black with a white stripe under her eye and, on some birds, red can be seen round the eye too. As with most hens, her colouring gives her excellent camouflage (Downie and Hunter, 2009).

Copper pheasant and domestic fowl (Gallus gallus domesticus) are the same order (Galliformes) and family (Phasianidae), but different genus and species. Unlike domestic fowl, the breeding season of copper pheasant is short with rapid and robust reproductive organs response at the onset of the season (from middle of March to the end of May in Miyazaki, Japan). Copper pheasants respond to the same semen collection and insemination techniques that are used for domestic fowl: i.e., the abdominal massage method is the most common method for semen collection. The quantity of semen ranged from 0.01 to 0.02 ml per ejaculation: rather small quantity compared with domestic fowl (0.5–0.8 ml), but more concentrated (approximately 10×10^9 cells/ml) than domestic fowl semen (approximately 3.5×10^9 cells/ml) (Maru et al., 1966; Smyth, 1968; Sugimoto et al., 2001).

Abundant evidence has shown that flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATP hydrolysis by dynein ATPase of the outer doublet microtubules (for review, see Satir, 1979; Warner and Mitchell, 1980; Gibbons, 1982; Mohri, 1993). Although this basic axonemal mechanism is fairly well understood, many factors and mechanisms of its regulation have still to be clarified. For example, unlike mammalian spermatozoa, domestic fowl spermatozoa display the characteristic phenomenon of reversible temperature-dependent immobilization: in simple salt solutions, they become immotile at the avian body temperature of 40–41 °C, but motility is restored by decreasing the temperature or by the addition of Ca^{2+} at 40 °C (Munro, 1938; Ashizawa and Nishiyama, 1978; Wishart and Ashizawa, 1987; Ashizawa et al., 1989a, 1994a; Thomson and Wishart, 1989, 1991). Ca^{2+} is the major stimulatory factor in body fluids such as seminal plasma, or the fluid of the female ovarian pocket taken at the time of ovulation, in which spermatozoa can maintain their motility even at 40 °C (Ashizawa and Wishart, 1987, 1992). The axoneme and/or accessory cytoskeletal components themselves appear to be directly involved in the temperature-dependent regulatory system, since the motility of demembranated spermatozoa is, as with intact spermatozoa, negligible at 40 °C and restored at 30 °C (Ashizawa et al., 1989a,b, 2000). However, the intracellular molecular mechanisms involved in immobilization and restoration of motility are still unknown.

From invertebrate to vertebrate species, intracellular cascade systems for the regulation of sperm motility are triggered by several factors, mainly second messengers such as Ca^{2+} and cyclic nucleotides, which seem to converge upon the phosphorylation and dephosphorylation of proteins via several kinds of protein kinases and phosphatases which regulate axonemal movement (for review, see Tash and Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Morisawa, 1994).

Even although copper pheasant and domestic fowl are the same order and family, no information is available concerning the characteristics of copper pheasant sperm motility, especially whether they display the same characteristic phenomenon of reversible temperature-dependent immobilization at body temperature as those of domestic fowl spermatozoa. If so, what kinds of factors are involved in the regulation of sperm motility? In the following experiment, therefore, attempts were made to investigate the effects of temperature on the motility of Ijima's copper pheasant spermatozoa compared with that of domestic fowl spermatozoa, using some stimulators and inhibitors of signal transduction pathways.

2. Materials and methods

2.1. Animals and preparation of spermatozoa

Ijima's copper pheasant males were obtained from eggs collected from the same nest of grassy hillside in Miyazaki Prefecture, Japan, and then incubated and hatched in incubator and raised for more than 1 year with permission from Miyazaki Prefectural government. Commercial White Leghorn roosters (Dekalb strain) were purchased from Amuse Poultry Breeding Farm, Miyazaki, Japan. All birds were housed in individual cages and fed ad libitum on a commercial breeder diet. Roosters were exposed to a photoperiod of 14 h light:10 h darkness, but copper pheasant males were exposed to natural conditions of light, so semen could be collected during only breeding season (from March to May).

Semen was collected by the method of Bogdonoff and Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately 10-fold in 150 mM NaCl with 20 mM TES (N-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 and centrifuged at 700 × *g* for 13 min at room temperature (20–25 °C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1×10^9 cells/ml.

2.2. Chemicals

Adenosine 5′-triphosphate (ATP), dibutyryl adenosine 3′:5′-monophosphate (diB-cAMP; a cell-permeable cAMP analog), dithiothreitol, 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7, a cell-permeable selective inhibitor of myosin light chain kinase), potassium glutamate, TES and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Calyculin A, a cell-permeable inhibitor of protein phosphatase-type 1 and -type 2A, was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

2.3. *Analysis of motility of spermatozoa*

The intact copper pheasant and domestic fowl sperm preparations were incubated aerobically in a water bath at 30 °C for 6 min, and then transferred to another water bath and incubated at 40 °C for 9 min. After that, they were re-incubated at 30 °C for 9 min. Finally, they were moved to 40 °C again and incubated for 9 min. Overall, the samples were incubated for 33 min, including two periods at each of 30 and 40 °C. During incubation, sperm motility were measured every 3 min at each temperature. The effects of the addition of CaCl$_2$, calyculin A, an inhibitor of protein phosphatase-type 1 and -type 2A, ML-7, a selective inhibitor of myosin light chain kinase (MLCK) and diB-cAMP were also examined at 30 and 40 °C to be compared to controls with no effectors. The inhibition constant (K_i) values of calyculin A for protein phosphatase-type 1 and -type 2A are around 2 and 0.5–1 nM, respectively (Ishihara et al., 1989) and the K_i value of ML-7 for MLCK is around 0.3 μM (Saitoh et al., 1987). Ordinarily, 10–100-fold higher concentrations are required for whole cells. Diluent for the measurement of intact sperm motility was TES/NaCl buffer described above.

The demembranated copper pheasant and domestic fowl sperm samples were also incubated at 30 then 40 °C, twice over and sperm motility was measured every 3 min at each temperature, as for intact spermatozoa. Demembranation and reactivation of spermatozoa were performed according to the method described previously (Ashizawa et al., 1989b). The extraction medium used consisted of 0.1% (v/v) Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM MgSO$_4$, 1 mM dithiothreitol and 20 mM Tris–HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM MgSO$_4$, 1 mM dithiothreitol, 20 mM Tris–HCl buffer (pH 7.9). To examine the effects of Ca^{2+} and MLCK, the time courses of motility following the addition of EGTA, CaCl$_2$ and ML-7 were also examined at 30 °C.

The suspension of spermatozoa was placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy (magnification on the 12-inch black and white monitor was approximately 600×) at 30 °C or 40 °C (Katz and Overstreet, 1981). Measurements were made on a total of 200–300 spermatozoa, distributed uniformly among three or more fields, to determine the percentage of vigorously motile spermatozoa. The percentage of vigorously motile spermatozoa was defined as 'motility' in this paper.

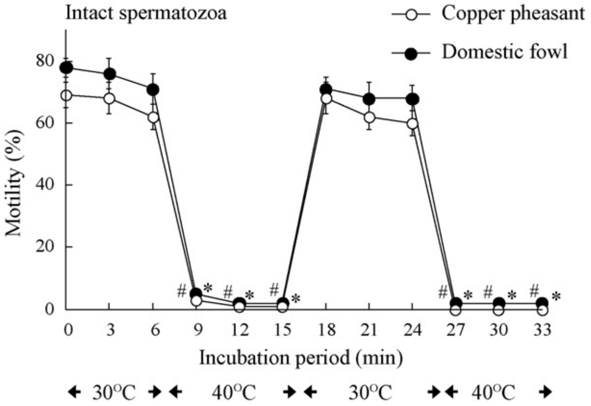

Fig. 1. The time course of motility of intact copper pheasant (○) and domestic fowl (●) spermatozoa during sequential incubation in TES/NaCl buffer (pH 7.4) at 30 °C (0–6 and 18–24 min) and 40 °C (9–15 and 27–33 min). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *#$P < 0.05$ compared with value at 0 min incubation of each spermatozoa.

2.4. *Statistical analysis*

All statistical analyses were carried out by the Statistical Analysis System R software package (R Development Core Team, 2008). For the evaluation of differences, the percentages of motility were analyzed by the generalized linear model (GLM, binomial error distribution) procedure followed by the Tukey test for non-parametric multiple comparisons (Ryan, 1960).

3. Results

3.1. *Effects of temperature on the motility of copper pheasant and domestic fowl spermatozoa*

Motility of both intact copper pheasant and domestic fowl spermatozoa was markedly affected by the temperature (Fig. 1). During the first incubation at 30 °C, copper pheasant spermatozoa showed around 60–70% motility, and even after a second incubation at 30 °C, a similar proportion of motile spermatozoa was found. In contrast, these spermatozoa lost their motility quickly at 40 °C. Temperature also affected the motility of demembranated copper pheasant spermatozoa as well as those of domestic fowl (Fig. 2). Motility was maintained vigorously at 30 °C but remained almost negligible at 40 °C, then when the temperature of the sperm suspension was subsequently cooled to 30 °C, the spermatozoa regained their motility.

3.2. *Effects of various effectors on the motility of copper pheasant and domestic fowl spermatozoa*

Fig. 3 shows sperm motility at 10 min after the addition of various effectors. The motility of intact copper pheasant and domestic fowl spermatozoa at 30 °C was unaffected 10 min after the addition of 2 mM CaCl$_2$, 100 nM calyculin A or 4 mM diB-cAMP, respectively, compared with controls with no effectors. However, the presence

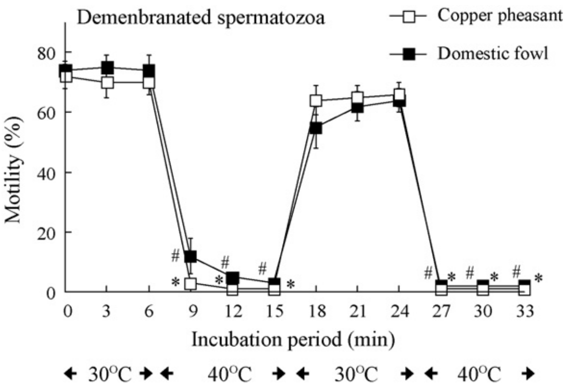

Fig. 2. The time course of motility of demembranated copper pheasant (□) and domestic fowl (■) spermatozoa during sequential incubation in the reactivation medium at 30 °C (0–6 and 18–24 min) and 40 °C (9–15 and 27–33 min). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *#$P < 0.05$ compared with value at 0 min incubation of each spermatozoa.

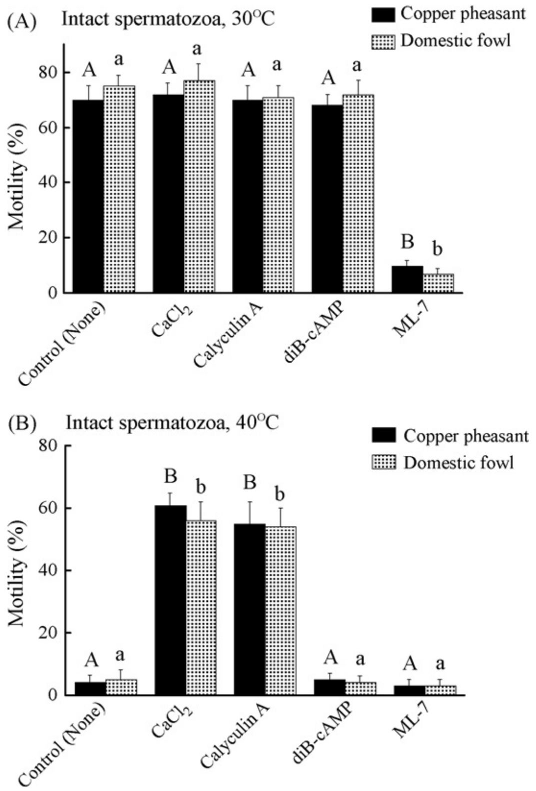

Fig. 3. Motility of intact copper pheasant and domestic fowl spermatozoa at 10 min after the addition of various effectors (2 mM $CaCl_2$, 100 nM calyculin A, 4 mM diB-cAMP and 10 μM ML-7) in TES/NaCl buffer (pH 7.4) at 30 °C (A) and 40 °C (B). Each value represents the mean (±S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.05$) from each other.

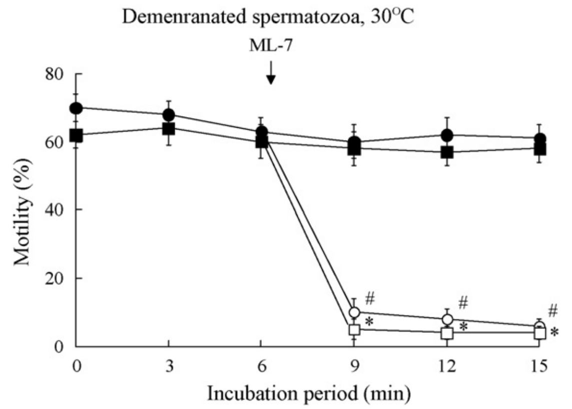

Fig. 4. The time course of motility of demembranated copper pheasant (■, □) and domestic fowl (●, ○) spermatozoa during incubation in the reactivation medium at 30 °C following addition of 10 μM ML-7 at arrow (○, □) or without ML-7 (control) (●, ■). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *#$P < 0.05$ compared with value when no inhibitor was added (control) at each period.

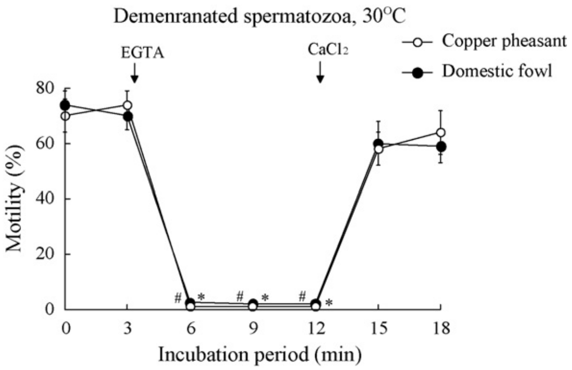

Fig. 5. The time course of motility of demembranated copper pheasant (○) and domestic fowl (●) spermatozoa during incubation in the reactivation medium at 30 °C following addition of 2 mM EGTA and 4 mM $CaCl_2$ at arrows. Each point represents the mean (±S.E.M.) of five samples of spermatozoa. $^{*\#}P < 0.05$ compared with value at 0 min incubation of each spermatozoa.

of 10 μM ML-7 inhibited copper pheasant and domestic fowl sperm motility significantly ($P < 0.05$) (Fig. 3A). At 40 °C, the motility of intact copper pheasant and domestic fowl spermatozoa was negligible. However, the presence of $CaCl_2$ or calyculin A permitted restoration of motility, but the addition of diB-cAMP or ML-7 could not prevent the immobilization of spermatozoa from either species (Fig. 3B).

The time courses of demembranated copper pheasant and domestic fowl motility at 30 °C, following various additions are shown in Figs. 4 and 5. In the presence of ATP, the motility of demembranated copper pheasant spermatozoa without ML-7 was over 60%. However, inhibition of motility of demembranated spermatozoa was observed following the addition of 10 μM ML-7. Similar effects were obtained for domestic fowl sperm motility (Fig. 4).

In the reactivation medium, $CaCl_2$ was not added at the beginning of incubation, but a ‘trace/contaminated’ calcium ion was present at around 100 nM, since EGTA was not added to the medium. Therefore, copper pheasant spermatozoa and domestic fowl spermatozoa were able to move in this condition. However, neither copper pheasant spermatozoa nor domestic fowl spermatozoa were motile following the addition of 2 mM EGTA to the reactivation medium. However, spermatozoa regained their motility after the subsequent addition of 4 mM $CaCl_2$ (Fig. 5).

4. Discussion

In general, it is expected that the rate of cellular functions such as motility should become faster as the temperature is raised. Whilst this is true for domestic fowl spermatozoa suspended in a simple salt solution as the temperature is raised from 20 to 35 °C, beyond this temperature sperm motility decreases, so that at the avian body temperature of 40–41 °C, the spermatozoa are completely immotile. This characteristic phenomenon was first reported by Munro (1938) who also noted that when the temperature of the sperm suspension was subsequently cooled to 30 °C, the spermatozoa regained their motility, so that the phenomenon is known as reversible, temperature-dependent motility immobilization (Munro, 1938; Ashizawa and Nishiyama, 1978; Ashizawa et al., 1989a).

The present study clearly showed that copper pheasant spermatozoa, the same order and family, but different genus and species from domestic fowl, display the same characteristic phenomenon as those of domestic fowl spermatozoa (Fig. 1). The pattern of temperature-dependent inhibition was also found in drake spermatozoa and, partially, in turkey spermatozoa. However, no such temperature-dependent inhibition was found in spermatozoa from Japanese quail and Houbara bustard (Wishart and Wilson, 1999). These observations suggest that such immobilization might not simply occur at the same classification in taxonomic ranks, since quail and domestic fowl/copper pheasant are the same order (*Galliformes*) and family (*Phasianidae*), and turkey is the same order (*Galliformes*) but different family (*Meleagrididae*) compared with domestic fowl/copper pheasant. On the other hand, drake (order: *Anseriformes*, family: *Anatidae*) and bustard (order: *Gruiformes*, family *Otididae*) are completely different from domestic fowl/copper pheasant. Difference in sperm motility profiles were also observed during sperm maturation in the testis of the domestic fowl, pigeon and drake on the one hand, and in the passerine song sparrow on the other. Domestic fowl spermatozoa, when removed from the testis were able to display some motility (Ashizawa and Sano, 1990). Testicular spermatozoa from pigeon and drake were also shown to have the capacity for motility, although not spermatozoa from the passerine song sparrow (Bedford, 1979). The proportion of intact testicular spermatozoa from the domestic fowl was shown to be around 75% of that of ejaculated spermatozoa and their velocity around 50%. However, for demembranated spermatozoa, the proportion of motile and velocity of testicular spermatozoa were each around 10% of ejaculated spermatozoa, suggesting that the immaturity of testicular spermatozoa resides mainly in their axonemal response to added ATP (Ashizawa and Sano, 1990).

If copper pheasant spermatozoa could remain motile at 40 °C after removing the plasma membrane, it was suspected that the plasma membrane and/or cytosol might be involved in the immobilization of copper pheasant spermatozoa at 40 °C. However, the motility of demembranated spermatozoa was, as with intact spermatozoa, almost negligible at 40 °C (Fig. 2). These results suggest that the mechanisms involved in the immobilization of copper pheasant spermatozoa at 40 °C do not involve the plasma membrane and/or cytosol, but the axoneme and/or accessory cytoskeletal components themselves as found for those of domestic fowl spermatozoa.

In the present study, when 2 mM $CaCl_2$ was added, the motility of intact copper pheasant and domestic fowl spermatozoa at 30 °C was unaffected, respectively, compared with the control (no addition of effectors) (Fig. 3A). However, at 40 °C, the presence of $CaCl_2$ permitted restoration of motility (Fig. 3B). Therefore, Ca^{2+} seems to play a key role in the stimulation of motility of copper pheasant and domestic fowl spermatozoa at the avian body temperature. Furthermore, the motility of intact domestic fowl spermatozoa was inhibited at both 30 and 40 °C by loading spermatozoa with the intracellular calcium chelator BAPTA/AM, but the inhibition could be released instantly by the addition of excess calcium to the medium (Ashizawa et al., 1994a). The locus of action of calcium is likely to be the axoneme and/or accessory cytoskeletal components, since the present study showed that in demembranated copper pheasant and domestic fowl spermatozoa at 30 °C, the addition of 2 mM EGTA to sequester 'contaminant' calcium, arrests the motility, and then spermatozoa regained their motility after the subsequent addition of 4 mM $CaCl_2$ (Fig. 5).

Substantial evidence implicates a major role for protein phosphorylation and dephosphorylation systems in the regulation of mammalian sperm movement, and the predominant system involves protein kinase A (PKA), which is dependent on the second messenger, cyclic AMP (cAMP), and has been identified in several species (for review, see Tash and Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Tash, 1989; Majumder et al., 1990; Morisawa, 1994). However, the addition of diB-cAMP could not prevent the immobilization of both copper pheasant and domestic fowl spermatozoa at 40 °C (Fig. 3B). Furthermore, the motility of demembranated domestic fowl spermatozoa at 40 °C could not be restored by the addition of cAMP (Ashizawa et al., 1989b, 1992). The addition of a PKA substrate peptide did not inhibit the motility of demembranated domestic fowl spermatozoa at 30 °C (Ashizawa et al., 1995a,b). Thus, it seems unlikely that the phosphorylation of protein(s) by PKA is involved in the maintenance of copper pheasant and domestic fowl sperm motility.

As described above, calcium seems to have significant effects on copper pheasant and domestic fowl sperm motility. Instead of PKA, axonemal and/or accessory cytoskeletal myosin light chain kinase (MLCK), or an MLCK-like protein, has been proposed to be a candidate of calcium-dependent protein kinase in domestic fowl spermatozoa, since the motility of both intact and demembranated domestic fowl spermatozoa at 30 °C decreased markedly following the addition of ML-9, a specific inhibitor of MLCK (Ashizawa et al., 1995b). Moreover, it may be possible that the phosphorylation of a 30-kDa protein which may be a substrate for MLCK or an MLCK-like protein might be necessary for the activation of domestic fowl sperm motility at 30 °C, since the addition of an MLCK substrate peptide decreased the phosphorylation levels of this protein and inhibited the motility of demembranated spermatozoa (Ashizawa et al., 1995a). It is interesting to note that in the present work, the presence of 10 μM ML-7, another specific inhibitor of MLCK, inhibited copper pheasant and domestic fowl sperm motility at 30 °C (Fig. 3A), and at 40 °C, the addition of ML-7 could not prevent the immobilization of both spermatozoa (Fig. 3B). Furthermore, inhibition of motility of demembranated copper pheasant and domestic fowl spermatozoa was observed following the addition of 10 μM ML-7 (Fig. 4). Thus, phosphorylation by MLCK or an MLCK-like protein might be necessary for the activation of not only domestic fowl spermatozoa, but also copper pheasant sperm motility.

If phosphorylation is required for the activation of flagellar motility, then dephosphorylation of proteins by specific regulatory phosphatases should reduce sperm motility. Such regulatory serine/threonine protein phosphatases are classified into four main types: PP1, PP2A, PP2B and PP2C (Cohen, 1989). With regard to dog spermatozoa, it has been proposed that inhibition of sperm motility by high concentrations of Ca^{2+} may be due to the activation of PP2B (calcineurin), a calcium-dependent protein phosphatase (Tash et al., 1988). In contrast, PP1 might be dominant in the temperature-dependent inhibition of flagellar movement of domestic fowl spermatozoa at 40 °C, since the motility of demembranated domestic fowl spermatozoa at 40 °C was stimulated by the addition of calyculin A or okadaic acid, specific inhibitors of PP1 and PP2A, and inhibitors 1 and 2, small heat-stable proteins which inhibit PP1 activity only (Ashizawa et al., 1994b). In addition, the motility of demembranated domestic fowl spermatozoa at 30 °C decreased markedly following the addition of recombinant PP1 supplemented with Mn^{2+}. This inhibition was not restored by the addition of cAMP (Ashizawa et al., 1997). The present study showed that the temperature-dependent immotility of copper pheasant spermatozoa at 40 °C was restored after the addition of calyculin A. These results suggest that PP1-mediated dephosphorylation of some proteins of copper pheasant spermatozoa may also be involved in the inhibition of motility.

In conclusion, copper pheasant sperm motility might be regulated by similar mechanisms to domestic fowl spermatozoa: i.e., the balance of Ca^{2+}/MLCK or an MLCK-like protein-dependent phosphorylation and PP1-dependent dephosphorylation. The similarity in physiological regulation of spermatozoa from both species suggests that extensive technology developed for artificial breeding of the domestic fowl might be applicable to captive breeding of Ijima's copper pheasants, one of the Japanese 'near threatened' species.

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